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Comparison of the Irritation Potency of Selected Wound Antiseptics in the Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) to Predict their Compatibility to Wounds

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1 Abstract

Introduction: Antiseptics are used for the prophylaxis of infections of acute wounds and for the treatment of critically colonized chronic wounds as well as localized infections of acute and chronic wounds. If an antiseptic with too much tissue toxicity and/or too little efficacy is used, the wound healing can be delayed.

Objective: The aim was to compare the irritation potency of frequently used wound antiseptics by using the hen's egg test on the chorioallantoic membrane (HET-CAM). Additionally, the influence of antiphlogistic active additives which might increase the tolerability was examined. To allow a more extensive comparison, antiseptics classified as obsolete such as hydrogen peroxide, creams on PVP- iodine base, silver sulfadiazine, chlorhexidine and nitrofural as well as the non-antiseptic wound treatment agents dexpanthenol and hemoglobin spray were also examined.

Method: The HET-CAM was used as a semi-in-vivo method to test the tolerability of wound antiseptics to tissues by observing the reactions that occur in the blood vessels of the highly vascularized CAM such as hemorrhage, lysis and coagulation. The irritation score (IS) was calculated and differentiated in 4 ranges according to Spielmann (1991).

Results: The vascular injuries of the CAM were considered as an indirect indicator of the tolerability. It is accepted that agents with no or low irritation potential on the CAM are to be preferred in the clinical practice if they are clinically effective.

Severe CAM reaction was observed after short-term application of octenidine based wound gel (active ingredient octenidine 0.05%) (IS: 10.3) and chlorhexidine digluconate 0.5% solution (IS: 9.5). Moderate reaction was observed for the combination of octenidine 0.05% in aqueous solution with panthenol 1.34% and allantoin 0.2% (IS: 8.7), hydrogen peroxide 1.5% in aqueous solution (IS: 6.1) and hydrogen peroxide 0.5% solution (IS: 5.5). Slight reaction was observed for hydrogen peroxide 1.5% solution in combination with sodium thiocyanate 0.698% (IS: 2.6), sodium thiocyanate 0.698% solution (IS: 2.1) and Dermacyn® (active ingredient NaOCl/HOCl each 0.004) (IS: 1.2). Polihexanide 0.04% in Ringer solution (IS: 0.9), Polihexanide 0.05% in Lipofundin, Granulox® (active agent hemoglobin 10%) (IS: 0) and dexpanthenol 5% solution (IS: 0) showed no reaction. In the long-term observation (24 hours after application), Dermacyn® showed the best results (59% of irritation remained alive after 24 hours). The addition of dexpanthenol and allantoin reduced the irritability only slightly, whereas the decrease of IS of hydrogen peroxide by addition of sodium thiocyanate was almost significant (p 0.0596).

Conclusion: It is suggested that agents with no or low irritation potential on the CAM are to be preferred in the clinical practice if they are clinically effective. It is suggested that further in vivo and in vitro studies are to be undertaken with these agents.

Solely regarding local tolerability, polihexanide and hypochlorite are the antiseptic agents of choice of the tested preparations. The wound oxygenizer hemoglobin spray is tolerated without irritation as well as the negative control 0.9% NaCl solution. Because of their other disadvantages in conjunction with their irritability, the outdated cream formulations on basis of silver sulfadiazine, PVP- iodine, chlorhexidine and nitrofural cannot be recommended for wound antisepsis.

Keywords: Wound antiseptics, HET-CAM, irritation score, octenidine, chlorhexidine, polihexanide, hypochlorite, hydrogen peroxide, hemoglobin spray, allantoin, dexpanthenol, thiocyanate.

2 Introduction

2.1 Aim

Antiseptic solutions, ointments and dressings are used for both prophylaxis of infections of acute wounds and treatment of critically colonized chronic wounds as well as localized infections of acute and chronic wounds (Kramer 2016, Kramer et al. 2013, 2016, 2017). Although antiseptics are used widely to make the healing time shorter, unfortunately, they may prolong the healing duration because of their cytotoxicity towards the wound tissue (Bolton et al. 1985, Kramer et al. 1998).

There is a question that I have personally encountered numerous times during my day to day work: Which antiseptic is the most tolerable one with the least irritation or side effects in short and long-term application? The aim of this study is to help finding answer to this question.

The intention is to create a comparison between the irritation potencies of selected wound antiseptics including the influence of antiphlogistic active additives, which may improve the tolerability, to give an option for further research.

A sensitive screening model to analyze the tolerability of wound antiseptics in vivo is the Hens' Egg test on the chorioallantoic membrane (HET-CAM) of chicken embryos. The HET-CAM was originally developed by Spielmann et al. (1991) to replace the Draize irritation test (Draize et al. 1944). In addition, the HET-CAM was used as a model for investigation of inflammation and angiogenesis (Dannhardt et al. 1996, Krenn and Paper 2009, Ribatti et al. 1996, Zwadlo-Klarwasser et al. 2001) as well as to test the mutagenicity (Kluge et al. 2016).

In the present work, the HET-CAM is used to test the tolerability of selected antiseptic active agents, commercially available wound antiseptics and possibly suitable additives for antiseptic formulations.

The planned study differs from other similar projects in the following points:

- The reliability of the sample means is higher, because the experiment was done on 9 eggs for every substance, while other published data had used only 3-6 eggs.
- The tolerability of the antiseptic to the tissue was observed 24 hours after application, which will help to recommend the less cytotoxic antiseptics for long exposure time.

- Although some of the antiseptics and formulations were tested before, the others are tested for the first time in this study.
- Some of the tested formulations are suggested mixtures of agents and additives, which aim to give an option for the development of new combinations with better tolerability.

2.2 The HET-CAM as a semi-in-vivo method to test the tolerability of wound antiseptics

The Draize irritation test on the eyes of rabbits, a clearly in vivo test method, can be considered as the first published test to evaluate the tolerability of substances to living tissues. In his publication "Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes", Draize et al. (1944) describes how to use the animals such as rabbits to measure the irritation potency of substances on the skin and mucous membranes.

The Draize irritation test was heavily criticized due to ethical reasons perspective because of the injuries of the tested animals. That is why several various tests have been developed to replace the rabbits in detecting the irritation potential of chemicals (Kishore et al. 2008).

One of the alternative tests is the HET-CAM (hen's egg test on the chorioallantoic membrane) which considered as an acceptable replacement scientifically and ethically. The HET-CAM avoids the ethical obstacle and allows enlarging the sample size.

The CAM is produced as an extraembryonic mesodermal double layer on the 4^{th}-6^{th} embryo development day from the fusion of chorion and allantois (Fuchs and Lindenbaum 1988, Bellairs and Osmond 1998). The ectodermal chorion surrounds the embryo and the yolk (Rizzo and DeFouw 1993). In the double membrane, a dense vascular network develops, which is connected to the embryo via arteries and veins (Rizzo et al. 1995). On the 10^{th} day of incubation, the capillary network overlays the area adjacent to the air bubble, which allows optical evaluation of the stimulus effect (DeFouw et al. 1989a, Fuchs and Lindenbaum 1988, Rizzo et al. 1995). During embryogenesis, the CAM can be found at the blunt pole of the egg near the air space. No nerve tissue develops in the chicken egg until the 11^{th} day, so no pain perception occurs at the time of the experiment (Liebsch and Spielmann 2002); thus, testing the chicken egg is not be classified as an animal experiment until the 11^{th} day of incubation (Bender et al. 2010).

The vascular system of the CAM reacts to harmful substances immediately; it reacts highly sensitively and can be evaluated visually. The CAM reacts to stimuli similar to the mammalian eye, therefore the HET-CAM is suitable for the investigation of irritation and inflammation reactions and is used as a predictive model for eye tolerance (Steiling et al. 1999), as model for tolerability to wounds (Ribatti et al. 1999, Ribatti et al. 1996a) and as a precursor for the assessment of wound healing (Kramer et al. 2013). Since no established animal model exists for chronic wounds, the HET-CAM at least allows for an orientating assessment of tissue tolerability. It can be assumed that antiseptic agents tolerated by the HET-CAM are compatible with the eye and wounds.

The first study to compare the irritation potential of antibiotic eye drops and the wound antiseptic polihexanide was done by Kramer and Behrens-Baumann (1997). Whereas polihexanide displayed no reaction on the CAM, the antibiotic eye drops (i.e. Posifenicol® and Dispaphenicol®) induced low to strong hyperemia and even sporadic hemorrhage. On this basis, it was possible to introduce the wound antiseptic polihexanide for preoperative eye antiseptic (Hansmann et al. 2004, 2005) and the surgical aphorism "do not apply anything into the wound that you cannot apply on eye" was deduced (Leaper et al. 2010, Assadian u. Kramer 2012, Kramer et al. 2013). From this point of view, the HET-CAM was used as a predictive test for the suitability of a treatment for wounds. Thus, the HET-CAM formed the decisive prerequisite for the introduction of cold atmospheric pressure plasma (CAPP) for wound treatment (Kramer et al 2013). Because the plasma is antiseptically effective, including activity against biofilms, and stimulates mild inflammation, chronic wounds could be successfully treated with the CAP in various pet species without negative influence on wounds (Bender et al. 2010, 2011, 2012, Bender and Kramer 2016). Thus, the HET-CAM allowed a prediction in order to apply plasma to wounds.

Considering the corresponding results of studies on the tolerability of antiseptics in cell culture (Müller and Kramer 2008), peritoneal explants (Kramer et al. 1998), HET-CAM (Kramer and Behrens-Baumann 1997, Reimer et al. 2000, Kramer et al. 2004, Roth et al. 2017), the HET-CAM was selected as a screening model for the tolerability of wound antiseptics to tissues in the present study. An additional aspect was that the HET-CAM, due to its high sensitivity, has a safety margin for the application of antiseptics on wounds (Scheel et al. 2011).

2.3 Overview of common wound antiseptics as a base for the selection of relevant test substances

In clinical practice, we choose the antiseptic which gives us the best results with the fewer side effects, so we give attention to the antiseptic's spectrum of activity, risk of development of resistance, cytotoxicity, absorption and systemic risks.

Summarizing, polihexanide, octenidine and the combination of sodium hypochlorite and hypochloric acid or sodium hypochlorite by itself are at present the antiseptics of choice for both acute and chronic wounds (Kramer 2016, Kramer et al. 2017). The only exceptions are bite and stab wounds; these wounds are to be treated with a combination of iodophors and alcohols because they have better tissue penetration (Kramer et al. 2012, 2017, Willy et al. 2017). Table 1 describes the most common wound antiseptics and compares between them.

Table 1: Brief characterization of antiseptic agents (modified after Kramer et al. 2012, 2017)

Active substance	Spectrum of activity	Development of resistance[1]	Remanent activity	Cytotoxicity[2]	Sensitization[3]	Absorption	Systemic risks	Biodegradability	Usage
Polihexanide	Broad spectrum, effective against biofilms	No hint	+	Promotion of wound healing	+[5]	-	-	Poor	First choice for chronic wounds and burns
Octenidine			+	Similar to Ringer's solution				Good	First choice for superficial acute wounds
Povidone iodine	Broad spectrum, loss of effectiveness in the presence of 20% blood		-	+	++	++	++	Poor (Povidone)	First choice antiseptic for stab and bite wounds
Medihoney®	Broad spectrum		-	-	+		-	Good	Special for children
Larvae	Broad spectrum		-	-	-			Not relevant	Special indications
Hypochlorite	Broad spectrum, effective against biofilms, bacterial spores and viruses		-	Promotion of wound healing	-	-	-	Good	Antiseptic cleaning of acute and chronic wounds
Silver ions	Low activity	+	+	+++	-	++	++	Poor	No first choice
Fusidic acid	Effective only against Gram-positives	++	?	++	++	+	?	Good	Not needed
Nitrofurazone	Gaps	+	?	++	++	+	+	?	Obsolete
Chlorhexidine	Gaps	++	+	+	+[6]	-	Possibly[4]	Good	Not needed
Hydrogen peroxide	Insufficient effective	-	-	++	-	-	-	Good	Obsolete

Continuation Table 1: Brief characterization of antiseptic agents (modified after Kramer et al. 2012, 2017)

Active substance	Spectrum of activity	Development of resistance[1]	Remanent activity	Cytotoxicity[2]	Sensitization[3]	Absorption	Systemic risks	Biodegradability	Usage
Ethacridine lactate	Insufficient effective	?	-	++	+ (airborne)[7]		?	?	Obsolete
Acetic acid	Broad spectrum	-	-	-	-	?	-	Good	Promising perspectives

[1] + in vitro ++ clinically relevant

[2] + low ++ moderate +++ high

[3] - very low risk + cause of contact eczemas in rare cases ++ cause of contact eczemas in many cases (>1%)

[4] the degradation product 4-chloroaniline are even carcinogenic, but its release up to now is demonstrated only in the mouth cavity (Below et al. 2017)

[5] Anaphylactic reactions (n = 3 worldwide; Kautz et al. 2010, Creytens et al. 2014, Schroeder et al. 2014)

[6] Anaphylactic reactions (1:10.000 – 1:25.000 after usage perioperatively; Moka et al. 2015)

[7] Rudzki and Rebandel (2001)

2.4 Field of applications for antiseptics

Antiseptics are needed to prevent and treat wound infections, especially when wounds are at increased risk of infection. Table 2 gives an overview of the risk factors for wound infection.

Table 2: Risk factors for wound infection (modified after Dissemond et al. 2011)

Wounds at particular risk of infection	
Endogenously and immunologically increased risk of infection	Exogenously increased risk of infection
Congenital immune defects	Heavily contaminated wounds (gunshot, bite, traumatic wounds)
Acquired immune defects	Presence of foreign bodies
Immunosuppressive medication	Postsurgical wounds following procedures with high microbial contamination
Diabetes mellitus	Specific pathogenicity and virulence of the pathogen present in the wound
Advanced age	Risk due to location (e.g. perineal surgery)
Young age (premature infants, babies, infants)	Environmental risks (e.g. occupational and lifestyle risks)
Burn wounds	smoking
Malnutrition	-
Blood flow problems	
Kidney diseases	
Obesity	

In 2011, the wound at risk score (W.A.R) was introduced as a practice oriented method to aid in deciding when to use antiseptics in order to prevent wound infections.

Based on the risk factors, the W.A.R. score (Table 3) can be calculated, a score ≥ 3 points represents a clinical indication for the administration of antiseptics. This is a pragmatic way to decide when the prophylactic application of antiseptics is indicated.

Table 3: Classification for wounds at risk: the W.A.R. score (from Dissemond et al. 2011)

Risk class	Risk definition (based on risk status and different indications)	Points (W.A.R.)
Class 1	a) Acquired immunosuppressive disease (e.g. diabetes mellitus) b) Acquired immune defect due to medical therapy such as cyclosporine, methotrexate, glucocorticoids or antibodies c) Solid tumor disease d) Systemic hematological disease e) Postsurgical wound healing disorder, which results in (unplanned) secondary healing f) Potentially heavily contaminated wounds (e.g. perineum, genitals) g) Problematic hygienic conditions related to social or occupational environment (e.g. agriculture, lorry driving) h) Patient age >80 years i) Young age of patient (premature infants, babies, infants) j) Wounds persisting for >1 year k) Wound dimensions of >10 cm^2 l) Chronic wounds of any etiology having a depth of >1.5 cm m) Extended inpatient status >3 weeks	The presence of each risk factor **adds 1 risk point** (multiple responses are possible) the points are added
Class 2	a) Severe acquired immune defects (e.g. HIV infection) b) Heavily contaminated acute wounds c) Bite, stab and gunshot wounds penetrating 1.5 - 3.5 cm	The presence of each risk factor **adds 2 risk points** (multiple responses are possible) the points are added
Class 3	a) Burn wounds with involvement of >15 % body surface area b) Wounds that have a direct connection to organs or functional structures (e.g. including joints) or which contain foreign material c) Severe congenital immune defects such as agammaglobulinemia, severe combined immune defects, etc. d) Bite, stab and gunshot wounds penetrating >3.5 cm e) contaminated soft tissue trauma	The presence of each risk factor **adds 3 risk points** (multiple responses are possible) the points are added
Finally, the risk factor points are added to obtain a total score. A **score ≥ 3 points** indicates the presence of a wound clinically at risk of infection and consequently represents a clinical indication for the administration of antiseptics. Regardless of this recommendation, other treatment indications may be present, which themselves require local antimicrobial treatment such as elimination of pathogens when multiply resistant pathogens are present specified by the Robert Koch Institute; critically colonized wounds.		

3 Methods and Materials

3.1 Safety and operating precautions

All procedures followed the institution's regulations and procedures for the handling of human or animal materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions were followed, including the use of laboratory coats, eye protection and gloves (ICCVAM 2010).

3.2 Materials

The following materials were been used:

- Vakzine Lohmann specific pathogen free eggs (VALO SPF, Lohmann GmbH Cuxhaven, Germany)
- Small-motored breeder (Typ KMB F/2, Ehret GmbH Emmendingen, Germany) with an automatic rotating mechanism and automatic humidity regulation (at 37 $\pm$ 1 °C und 55 $\pm$ 7 % relative humidity)
- Egg Candler (Powerflash, J. Hemel Brutgeräte GmbH & Co.KG Verl, Germany)
- Scissors
- Handheld digital microscope (model 44300, Celestron, Torrance, CA, U.S.A., up to 400x magnification),
- Camera (Canon Power Shot G9)
- Stop clock
- Cold-light lamp
- pH-meter
- Thermometers
- Tapered forceps and tweezers
- Pipettes (300 µl)
- 0.1 n NaOH in distilled sterile water (positive standard I)
- 1% Sodium dodecyl sulfate (SDS) solution in distilled sterile water (positive standard II)
- 0.9% NaCl solution in distilled sterile water
- Polyethylene film
- Fume cupboard

The following antiseptics and formulations were tested:

- Octenilin® wound irrigation (Schülke GmbH, Norderstedt, Germany): Active agent octenidine hydrochloride 0.05% (ingredients: Aqua valde purficata, glycerol, ethylhexylglycerol)
- Octenidin 0.05% (Schülke GmbH, Norderstedt, Germany) in aqueous solution + D-panthenol (Bayer Vital GmbH, Leverkusen) 1.34% + allantoin (Merck KGaA Darmstadt, Germany) 0.2%
- Dermacyn® Wound care solution (SastoMed GmbH Georgsmarienhütte, Germany): Active agent NaOCl/HOCl (each 0.004%)
- Granulox® spray (SastoMed GmbH Georgsmarienhütte, Germany): Active agent hemoglobin 10% (ingredients: phenoxyethanol 0.1%, sodium chloride 0.9%, N-acetylcystein 0.5% in aqueous solution)
- Furacin® sol 0.2% ointment (Riemser Arzneimittel AG Riems, Germany): Active agent nitrofural 0.2%
- Polihexanide (Fagron Services BV, Uitgeest, Netherlands; Manufacturer Arch UK Biocides, UK) in Ringer solution (Fresenius Kabi GmbH Bad Homburg, Germany): Active agent polihexanide 0.04%
- Polihexanide (Fagron Services BV, Uitgeest, Netherlands; Manufacturer: Arch UK Biocides, UK): 0.05% in Lipofundin® o/w emulsion (B. Braun Melsungen AG) (mixture of both components in the laboratory)
- Braunovidon® ointment (B. Braun Melsungen AG, Germany): Active agent: Povidone iodine 10%
- Flammazine® cream (Sinclair Pharma GmbH Frankfurt am Main, Germany): Active agent silver sulfadiazine 1%
- Chlorhexidine digluconate 0.5% solution in sterile aqueous solution (mixture of both components in the laboratory)
- Bepanthen® antiseptic wound cream (Bayer Vital GmbH, Leverkusen, Germany): Active agent chlorhexidine digluconate 0.5%, (ingredients: Dexpanthenol 5%)
- Dexpanthenol 5% (Bayer Vital GmbH, Leverkusen, Germany) in sterile aqueous solution (mixture of both components in the laboratory)
- Sodium thiocyanate (Fluka Chemie GmbH über Sigma-Aldrich GmbH, Steinheim, Germany, Germany): 0.698% solution in aqueous solution (mixture of both components in the laboratory)
- Hydrogen peroxide (Fluka Chemie GmbH (Buchs, Switzerland über Sigma-Aldrich Chemie GmbH Steinheim, Germany): 0.5% in aqueous solution (mixture of both components in the laboratory)

- Hydrogen peroxide (Fluka Chemie GmbH Buchs, Switzerland über Sigma-Aldrich Chemie GmbH, Steinheim, Germany): 1.5% in aqueous solution (mixture of both components in the laboratory)
- Hydrogen peroxide (Fluka Chemie GmbH Buchs, Switzerland über Sigma-Aldrich Chemie GmbH, Steinheim, Germany): 1.5% + sodium thiocyanate 0.698% in aqueous solution (mixture of both components in the laboratory

0.1 n NaOH in distilled sterile water and 1% Sodium dodecyl sulfate (SDS) solution in distilled sterile water were tested as positive controls, 0.9% NaCl solution in distilled sterile water was tested as negative control.

3.3 Study protocol

This study used the test protocol used in Phase II of the German Validation Study for Replacement of the Draize Eye Test (Spielmann and Liebsch 1991).

Incubation of eggs:

- After the arrival of the eggs, they are placed in a warming box for 48 h at 15 °C (± 1 °C). Before insertion into the box, the eggs are disinfected by a wipe soaked with 70% v/v ethanol (produced in the pharmacy of the University of Greifswald, Germany). Additionally, the eggs are weighed and numbered.
- As next step, the eggs are candled and discarded in case of defects.
- Before incubation, the incubator's (KMB F/2, Ehret GmbH Emmendingen, Deutschland) reservoir was filled with water. The eggs were placed flat onto incubator trays in a 37.5 ± 1°C and 62 ± 7.5% rel. humidity, and were rotated automatically for 8 days to prevent the attachment of the embryo to one side of the egg.
- The temperature of the incubator was controlled automatically, humidity and water level were checked manually every day.
- The eggs were candled on the ninth day with Powerflash (J. Hemel Brutgeräte GmbH & Co.KG Verl, Germany), and nonviable eggs were discarded; then the fertilized eggs were placed in the incubator with the large end upward without rotation, thus ensuring accessibility to the chorioallantoic membrane.
- On day 10, the eggs were prepared for the test.

Preparation of eggs:

- Each egg was candled before preparation to ensure that all are viable, a cold lamp was used to ensure optimal illumination of the chorioallantoic membrane. any nonviable egg was excluded.
- In a fume cupboard, the air cell was marked using an Egg Candler and then the shell was removed off by the scissors.
- The membrane was carefully moistened with 0.9% NaCl solution at 37 °C.
- The eggs were placed in the incubator until they were ready for being tested.
- Fresh standards and test substances were prepared (in the appropriate solvents) before each test cycle at room temperature.

Test procedure and assessment:

- The opened egg was taken out of the incubator, then the moistening solution (0.9% NaCl) was poured off, and carefully the membrane was removed (without injuring any underlying blood vessels) using tapered forceps.
- A photo (Canon Power Shot G9 Camera) for the (CAM) was taken before adding the test substance.
- 0.3 ml of each standard or test substance was applied onto the CAM.
- The reactions on the CAM were observed over a period of 5 minutes, what was monitored is the appearance of:
    - Hemorrhage (bleeding).
    - Vascular lysis (blood vessel disintegration).
    - Coagulation (protein denaturation intra- and extravascular).
- The time in seconds for each reaction to occur was recorded and then the irritation score (IS) was calculated using the following formula:

$$IS = \left(\frac{301-t(h)}{300}\right) \times 5 + \left(\frac{301-t(l)}{300}\right) \times 7 + \left(\frac{301-t(c)}{300}\right) \times 9$$

h = hemorrhage; l = vessel lysis; c = coagulation; t = start second

- The mean of the irritation score (IS) for all the tested eggs was calculated. The IS encompasses values between 0 and 21. The effects of vascular irritation are measured semi-quantitatively and are differentiated in 4 (IS) ranges according to Spielmann (Marquardt et al. 2010):

    0 = no reaction (0.0 – 0.9)

    1 = slight reaction (1.0 – 4.9)

    2 = moderate reaction (5.0 – 8.9)

3 = severe reaction (9 – 21)

- Another photo for the (CAM) was taken 5 minutes after adding the test substance.
- After the exposure time of 5 minutes, the main reaction (either hemorrhage = bleeding; vascular lysis = blood vessel disintegration; or coagulation = protein denaturation intra- and extravascular) was recorded with reaction severity according to the following scheme:

  0 = No reaction

  1 = Slight reaction

  2 = Moderate reaction

  3 = Severe reaction
- When the test substance was a cream, an ointment, or a colored solution that disabled the monitoring of the CAM and impedes calculating the IS, the test followed the following algorithm: In case of cream or ointment, the cream or ointment was put on a plastic slide, the slide was applied onto the CAM for 5 minutes and then removed. In case of solution, the solution was put onto the CAM to cover approximately half of its surface, and after 5 minutes, the solution was carefully removed using a sterilized tissue to absorb the solution, and then the main reaction (hemorrhage, lysis, coagulation) was recorded and scored according to the same previous scheme (see above).
- The mean of the reaction severity after 5 min for all tested eggs was calculated.
- Afterwards the eggs were covered with an adhesive polyethylene film and incubated for another 24 hours. The CAM of each egg was then re-evaluated to see which of the embryos remained alive after 24 hours.
- Photos of the living embryos were taken after 24 hours.
- At the end of the test the embryos were killed as quickly as possible (by placing the eggs into a freezer at -20 °C).

Any shaking, unnecessary tilting, knocking and all other mechanical irritation of the eggs was avoided.

Test scheme:

The irritation score (IS) was determined for the two positive standards with two to three eggs at each day of the experiment. The test protocol specifies: The 1% SDS solution should give an IS of 10 ± 2, the 0.1 n NaOH solution an IS of 15 ± 3. 1% SDS should show hemorrhage and lysis within the first minute, whereas 0.1 n NaOH shows all 3

phenomena, first hemorrhage within several seconds, later coagulation and lysis at about 1 minute (Spielmann and Liebsch 1991).

3.4 Experiment phases

Tests were done in 3 phases (Table 4). In each round 90 eggs were tested, so the total number of eggs used in the experiments was 270. 64 eggs were excluded because they were nonviable, or broken, or the chorioallantoic membrane was severely injured during the experiment.

Table 4: Experiment phases

Round	Experiment day	Number of tested eggs
First	1	30
	2	30
	3	30
Second	4	45
	5	45
Third	6	45
	7	45

3.5 Contamination test

After finishing the second phase of the experiment, it was noticed that the majority of the eggs died after 24h even though they were being incubated. Therefore, multiple samples (n = 6) of the surface of the CAM were taken using cotton swabs to check bacterial contamination and then:

- The samples were spread on the surfaces of different blood agar dishes
- Each Petri dish was labeled with the source of the sample
- The Petri dishes were placed in a microbiological incubator for 48 h at 36 $\pm$ 2 °C
- Finally, the colony forming units (cfu) were recorded.

The aim was to detect if the death of the embryos was caused by contamination or not.

3.6 Statistical analysis

Statistical analysis was made and plots were calculated using SAS (Statistical Analysis Software, SAS Institute Inc., Cary, USA). Data (IS) were presented as mean with standard deviation and minimal and maximal value of IS. Descriptive statistics included median, 25th and 75th percentiles. The values were tested for normal distribution.

The significances were tested in comparison to the controls 0.1 N NaOH resp. 1% SDS as well as between the different test groups.

Statistical analysis was done using Graph Pad Prism Version 6.0. Descriptive statistics included mean, median, maximum, minimum, 25th and 0.75th percentiles and standard deviation. Normal distribution of the values was confirmed using D'Agostino-Pearson omnibus K2 test. Significant differences were calculated with ordinary one-way ANOVA followed by Dunnett's multiple comparisons test to compare test groups with positive control, 0.1 N NaOH or 1% SDS respectively, and by Tukey's multiple comparisons test to calculate differences between test groups.

4 Results

4.1 Irritation Scores in the HET-CAM

In the negative controls with 0.9% NaCl in distilled sterile water no reaction was induced in any case within 5 minutes (Table 5); thus, the irritation score (IS) was zero. 10 of 18 eggs died after 24 hours; on alive eggs (8 of 18) also no reaction was observed.

Table 5: Results of negative control (0.9% NaCl solution in distilled sterile water)

Day*	Egg	Time (s) for reaction to occur			IS	After 5 min		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Severity	
1	1	No reaction	No reaction	No reaction	0	No reaction	0	Alive
1	2				0		0	Died
1	3				0		0	Alive
2	4				0		0	Died
2	5				0		0	Alive
2	6				0		0	Died
3	7				0		0	Alive
3	8				0		0	Died
4	9				0		0	Died
4	10				0		0	Died
4	11				0		0	Died
5	12				0		0	Died
5	13				0		0	Alive
6	14				0		0	Alive
6	15				0		0	Alive
6	16				0		0	Died
7	17				0		0	Died
7	18				0		0	Alive
Number of eggs tested: **18**								
Mean of irritation score (IS): **0**								
Standard deviation of irritation score (IS): **0**								
Irritation score (IS) range according to Spielmann: **No reaction**								
Most common main reaction after 5 minutes: **No reaction**								
Mean of the reaction severity after 5 minutes: **0**								
Percentage of alive eggs after 24 hours: **44%**								
*The controls were carried out on different days.								

In figure 1, CAM is presented before application of the control solution, the blood vessels are normal without hemorrhage, lysis or coagulation. The CAM in figure 2 after application of the negative control solution does not differ from the initial state.

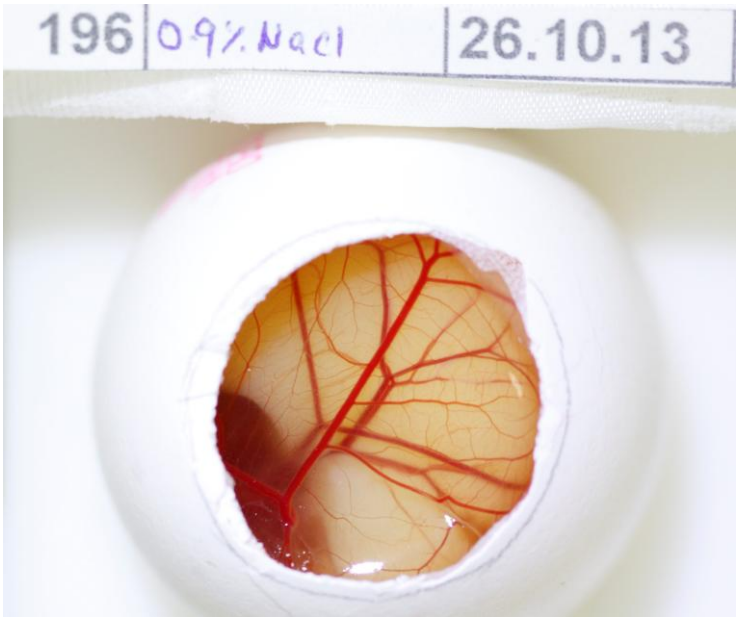

Figure 1: CAM before adding negative control (0.9% NaCl in distilled sterile water)

Figure 2: No reaction is noticeable on CAM 5 minutes after applying negative control (0.9% NaCl in distilled sterile water)

In table 6, the reactions of each egg (n = 18) of positive controls (0.1 n NaOH in dist. sterile water) are presented. The mean of IS was 15.3, the most common main reaction after 5 minutes was hemorrhage, no egg survived after 24 hours. The findings according to the test protocol: 0.1 n NaOH in distilled water should give an IS of 15 ± 3 and should show all 3 phenomena hemorrhage, coagulation and lysis.

Table 6: Results of positive control with 0.1 n NaOH in distilled sterile water

Day	Egg	Time (s) for reaction to occur			IS	After 5 min		After 24 hours
		Hemorrhage	Lysis	Coagu-lation		Main reaction	Se-verity	
1	1	13	150	34	16.3	Hemorrhage	3	Died
1	2	16	150	301	8.2	Hemorrhage	2	Died
1	3	9	130	107	14.6	Hemorrhage	2	Died
2	4	9	100	137	14.4	Hemorrhage	3	Died
2	5	11	120	132	14.1	Hemorrhage	3	Died
2	6	19	75	110	15.7	Hemorrhage	3	Died
3	7	6	148	90	14.8	Hemorrhage	3	Died
3	8	17	133	110	14.3	Hemorrhage	3	Died
4	9	10	200	50	14.7	Hemorrhage	3	Died
4	10	9	90	55	17.1	Hemorrhage	3	Died
5	11	11	77	53	17.5	Hemorrhage	3	Died
5	12	6	60	66	17.5	Hemorrhage	3	Died
5	13	9	44	86	17.3	Hemorrhage	3	Died
6	14	13	70	150	14.7	Hemorrhage	3	Died
7	15	16	16	140	16.2	Hemorrhage	3	Died
7	16	15	43	120	16.2	Hemorrhage	3	Died
7	17	15	50	130	15.7	Hemorrhage	3	Died
7	18	15	52	135	15.5	Hemorrhage	3	Died
Number of eggs tested: **18**								
Mean of irritation score (IS): **15.3**								
Standard deviation of irritation score (IS): **2.1**								
Irritation score (IS) range according to Spielmann: **Severe reaction**								
Most common main reaction after 5 minutes: **Hemorrhage**								
Mean of the reaction severity after 5 minutes: **3**								
Percentage of alive eggs after 24 hours: **0%**								

In figures 3 and 4, a typical example of CAM before and after application of 0.1 n NaOH can be seen.

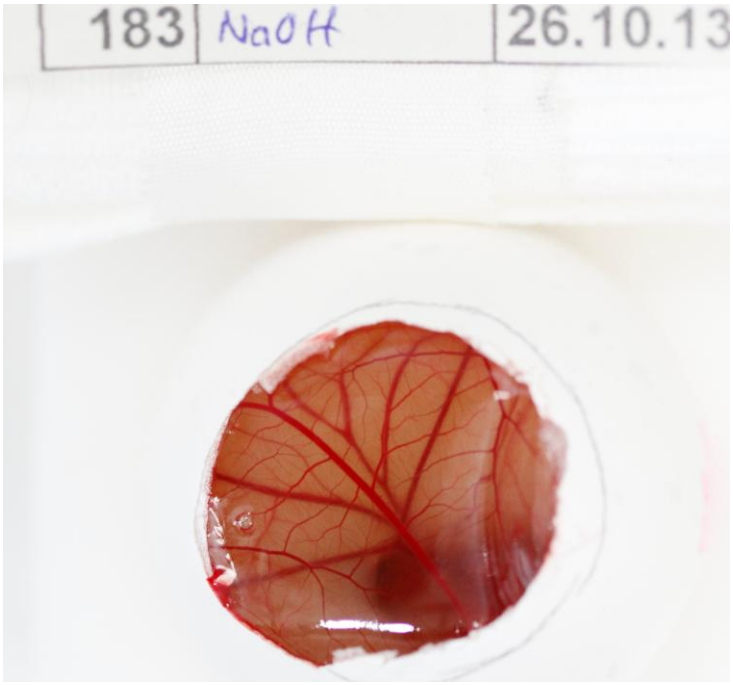

Figure 3: CAM before adding 0.1 n NaOH in distilled sterile water

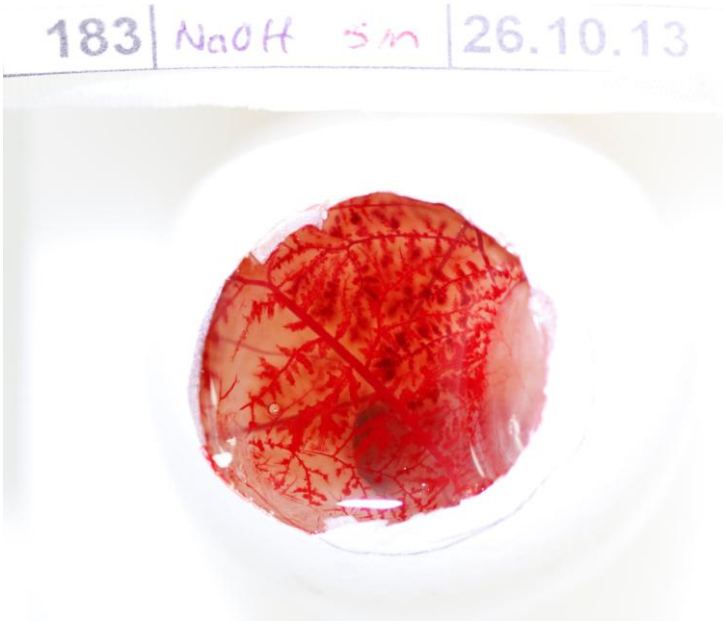

Figure 4: CAM shows hemorrhage, lysis and coagulation 5 minutes after adding 0.1 n NaOH in distilled sterile water

According to the test protocol, 1% SDS should give an IS of 10 ± 2 and should show hemorrhage and lysis. The mean of IS at the experiment was 10.0, and all the 18 eggs show hemorrhage and lysis (Table 7).

Table 7: Results of positive control with 1% SDS solution in distilled sterile water

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
1	1	21	81	No reaction	9.8	Hemorrhage	1	Died
1	2	25	90	No reaction	9.5	Lysis	2	Died
1	3	90	245	No reaction	4.8	Hemorrhage	1	Died
2	4	21	112	No reaction	9	Lysis	2	Died
2	5	65	123	No reaction	8	Lysis	1	Died
2	6	85	129	No reaction	7.6	Lysis	2	Died
3	7	27	160	80	14.4	Hemorrhage	1	Died
3	8	50	107	No reaction	8.7	Lysis	1	Died
4	9	22	155	No reaction	8	Hemorrhage	2	Died
4	10	43	205	102	12.5	Lysis	2	Died
5	11	34	90	No reaction	9.3	Lysis	2	Died
5	12	26	79	125	15	Lysis	2	Died
5	13	33	67	No reaction	9.9	Hemorrhage	1	Died
6	14	22	53	No reaction	10.4	Lysis	2	Died
7	15	20	30	No reaction	11	Lysis	2	Died
7	16	23	40	No reaction	10.7	Lysis	2	Died
7	17	20	60	No reaction	10.3	Lysis	2	Died
7	18	15	40	No reaction	10.8	Lysis	2	Died
Number of eggs tested: **18**								
Mean of irritation score (IS): **10.0**								
Standard deviation of irritation score (IS): **2.39**								
Irritation score (IS) range according to Spielmann: **Severe reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **1.66**								
Percentage of alive eggs after 24 hours: **0%**								

Before application of 1% SDS solution, the CAM was normal without any deviation (Figure 5). After application of 1% SDS solution, the CAM reacts with hemorrhage and lysis (Figure 6).

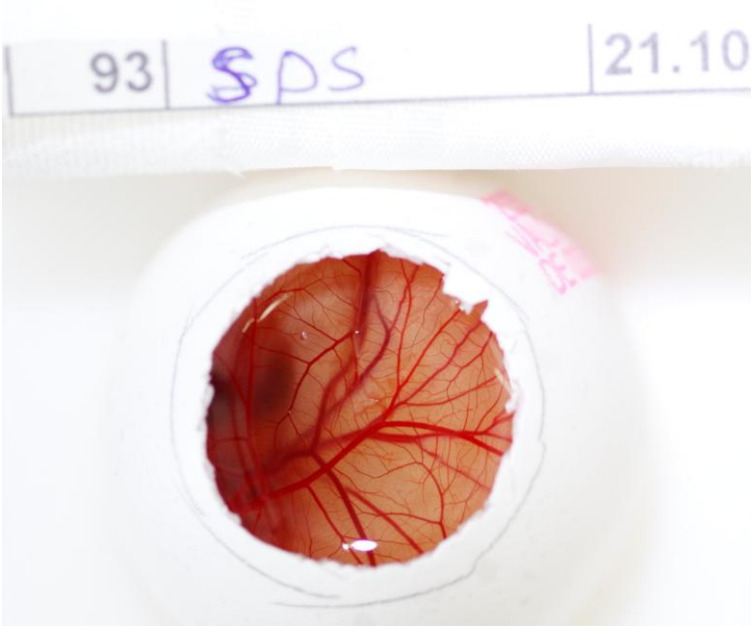

Figure 5: CAM before adding 1% SDS solution in distilled sterile water

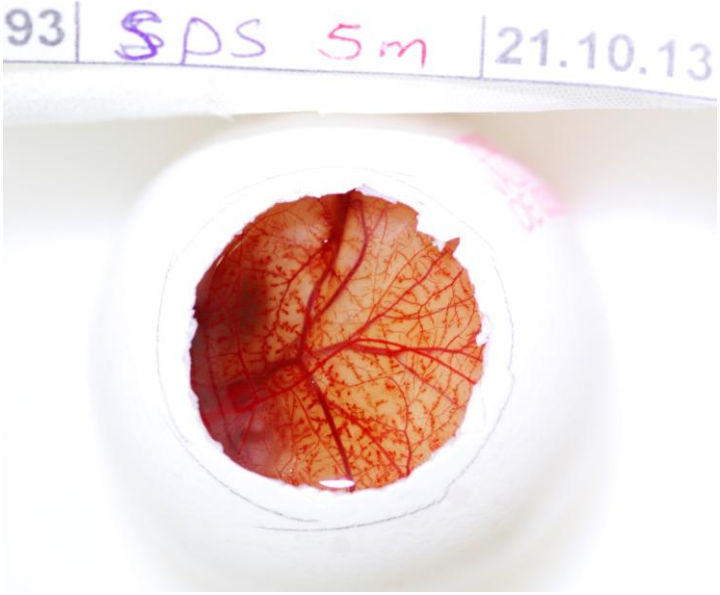

Figure 6: CAM shows hemorrhage and lysis 5 minutes after adding 1% SDS solution in distilled sterile water

The wound irrigation solution Octenilin® induced severe reactions. After 5 minutes, 6 eggs reacted with coagulation of severity 2, 3 eggs with lysis of severity 1 (Table 8).

Table 8: Irritation Potency of Octenilin®

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
1	1	150	No reaction	25	10.7	Coagulation	2	Died
1	2	250	280	15	9.9	Coagulation	2	Died
1	3	153	205	29	12.8	Coagulation	2	Died
2	4	110	44	89	15.5	Coagulation	2	Died
2	5	No reaction	200	93	8.5	Coagulation	2	Died
2	6	67	160	105	13	Coagulation	2	Died
3	7	No reaction	166	180	6.7	Lysis	1	Died
3	8	No reaction	175	240	4.7	Lysis	1	Died
7	9	50	50	No reaction	10	Lysis	1	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **10.2**								
Standard deviation of irritation score (IS): **3.33**								
Irritation score (IS) range according to Spielmann: **Severe reaction**								
Most common main reaction after 5 minutes: **Coagulation**								
Mean of the reaction severity after 5 minutes: **1.66**								
Percentage of alive eggs after 24 hours: **0%**								

In figure 7, the CAM before application of Octenilin® is seen; 5 minutes after application of Octenilin®, the CAM shows hemorrhage and lysis (Figure 8).

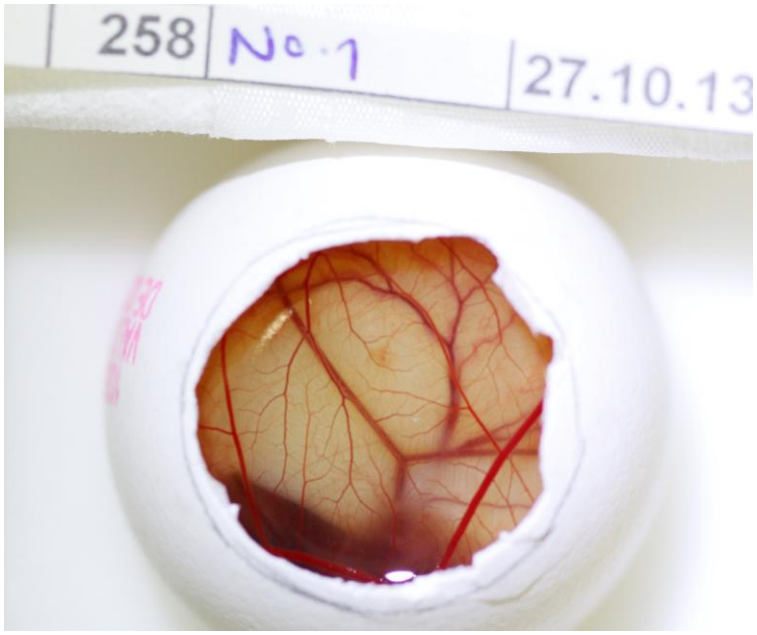

Figure 7: CAM before adding Octenilin® solution

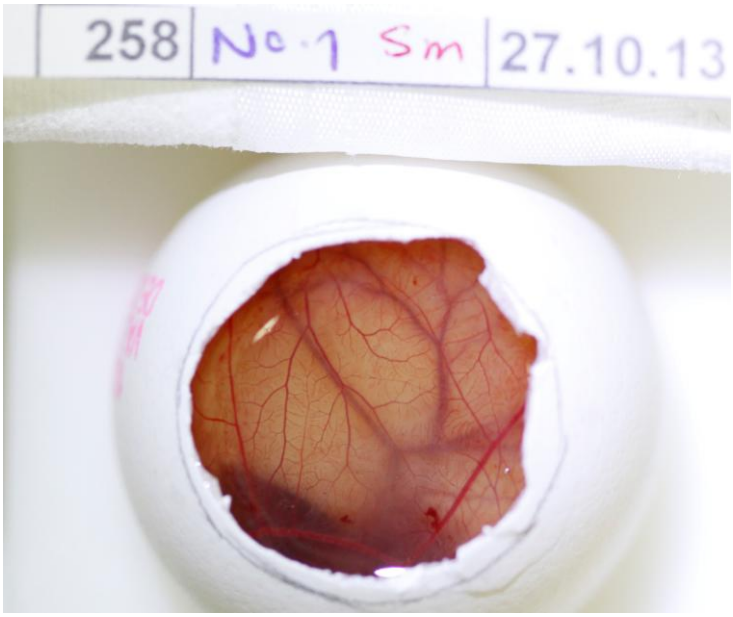

Figure 8: CAM with hemorrhage and lysis 5 minutes after adding Octenilin® solution

The combination of octenidine with dexpanthenol and allantoin showed all reaction types, this means hemorrhage, coagulation and lysis with severity ranged between 1 and 3. The mean of IS was 8.7.

Table 9: Irritation potency of octenidine 0.05% in aqueous solution + dexanthenol 1.34% + allantoin

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
5	1	164	140	No reaction	6	Hemorrhage	1	Died
5	2	154	110	120	12.3	Coagulation	1	Died
5	3	215	126	No reaction	5.5	Lysis	1	Died
5	4	120	166	No reaction	6.1	Hemorrhage	1	Died
7	5	60	34	No reaction	10.2	Lysis	2	Died
7	6	105	35	No reaction	9.4	Lysis	3	Died
7	7	73	39	No reaction	9.9	Lysis	3	Died
7	8	94	38	No reaction	9.5	Lysis	2	Died
7	9	123	27	No reaction	9.3	Lysis	1	Died
Number of eggs tested: **9 eggs**								
Mean of irritation score (IS): **8.7**								
Standard deviation of irritation score (IS): **2.3**								
Irritation score (IS) range according to Spielmann: **Moderate reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **1.66**								
Percentage of alive eggs after 24 hours: **0%**								

Figure 9 shows the CAM before the application of the mixture of octenidine with addition of dexpanthenol and allantoin. Figure 10 gives an example of moderate reaction after 5 minutes.

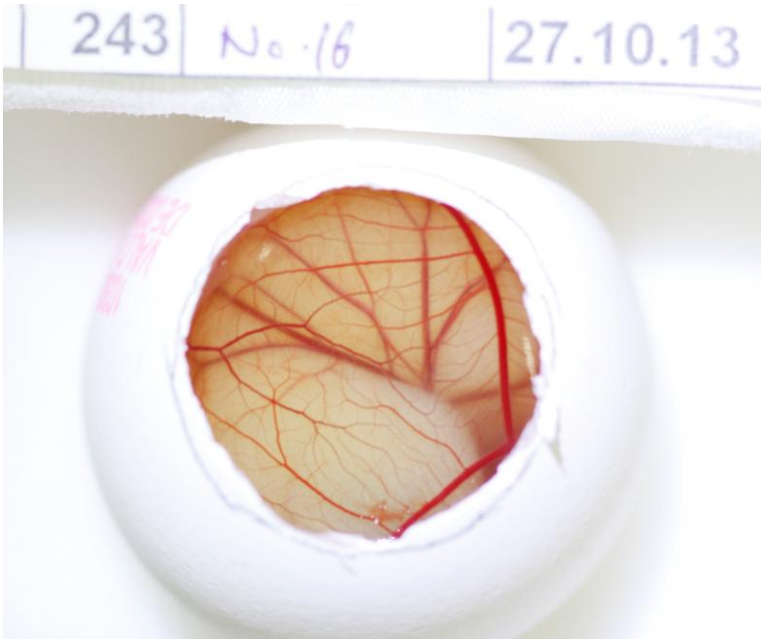

Figure 9: CAM before adding octenidine 0.05% + dexpanthenol 1.34% + allantoin 0.2% in aqueous solution

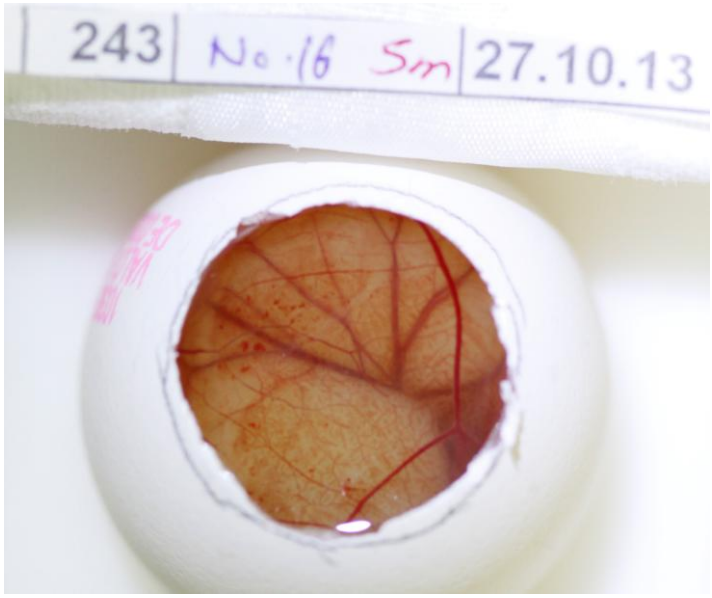

Figure 10: CAM shows hemorrhage and lysis 5 minutes after adding octenidine 0.05% + dexpanthenol 1.34% + allantoin 0.2% in aqueous solution

After application of Dermacyn® Wound care solution, 9 eggs showed no reaction. The other 8 eggs reacted within 5 minutes with lysis of severity 1 or 2 (Table 10). The mean of IS was 1.1, the IS range was considered as “slight reaction”.

Table 10: Irritation Potency of Dermacyn® Wound care solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
1	1	No reaction	220	No reaction	1.8	Lysis	1	Died
1	2	No reaction	285	No reaction	0.3	Lysis	1	Alive
1	3	No reaction	No reaction	No reaction	0	No reaction	0	Alive
1	4	No reaction	156	No reaction	3.3	No reaction	0	Died
2	5	No reaction	280	No reaction	0.4	Lysis	1	Died
2	6	No reaction	No reaction	No reaction	0	No reaction	0	Alive
3	7	No reaction	No reaction	No reaction	0	No reaction	0	Alive
3	8	No reaction	No reaction	No reaction	0	No reaction	0	Alive
6	9	No reaction	260	No reaction	0.9	Lysis	1	Alive
6	10	No reaction	No reaction	No reaction	0	No reaction	0	Alive
6	11	No reaction	No reaction	No reaction	0	No reaction	0	Died
6	12	No reaction	No reaction	No reaction	0	No reaction	0	Alive
6	13	No reaction	200	No reaction	2.3	Lysis	1	Alive
7	14	No reaction	230	No reaction	1.6	Lysis	1	Died
7	15	No reaction	90	No reaction	4.9	Lysis	1	Died
7	16	No reaction	No reaction	No reaction	0	No reaction	0	Alive
7	17	No reaction	150	No reaction	3.5	Lysis	2	Died
Number of eggs tested: **17**								
Mean of irritation score (IS): **1.1**								
Standard deviation of irritation score (IS): **1.54**								
Irritation score (IS) range according to Spielmann: **Slight reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **1**								
Percentage of alive eggs after 24 hours: **59%**								

The CAM before application of Dermacyn® (Figure 11) did not differ in 9 eggs from the clinical picture after application. In figure 12 a CAM after application of Dermacyn® Wound care solution is selected.

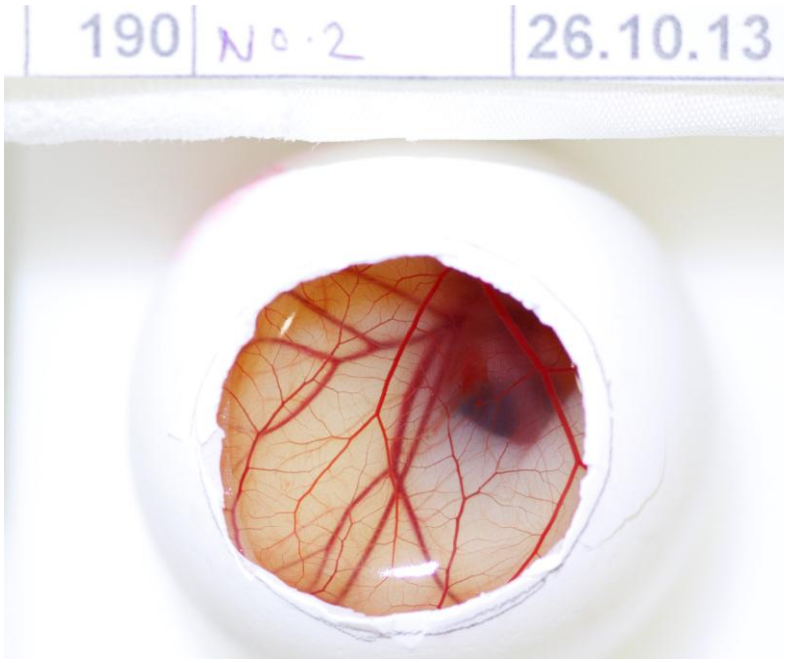

Figure 11: CAM before adding Dermacyn® Wound care solution

Figure 12: CAM shows no reaction 5 minutes after adding Dermacyn® Wound care solution

Although Granulox® spray is red, it allows the monitoring of the reactions happening on the CAM. Of 9 eggs, no CAM showed any reaction (Table 11). After 24 hours, no egg was alive.

Table 11: Irritation potency of Granulox® spray

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
1	1	No reaction	No reaction	No reaction	0	No reaction	0	Died
1	2	No reaction	No reaction	No reaction	0	No reaction	0	Died
1	3	No reaction	No reaction	No reaction	0	No reaction	0	Died
2	4	No reaction	No reaction	No reaction	0	No reaction	0	Died
2	5	No reaction	No reaction	No reaction	0	No reaction	0	Died
2	6	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	7	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	8	No reaction	No reaction	No reaction	0	No reaction	0	Died
7	9	No reaction	No reaction	No reaction	0	No reaction	0	Died
Number of eggs tested: **9 eggs**								
Mean of irritation score (IS): **0**								
Standard deviation of irritation score (IS): **0**								
Irritation score (IS) range according to Spielmann: **No reaction**								
Most common main reaction after 5 minutes: **No reaction**								
Mean of the reaction severity after 5 minutes: **0**								
Percentage of alive eggs after 24 hours: **0%**								

One example of a CAM before (Figure 13) and after application of Granulox® spray (Figure 14) is given. There was no difference after 5 minutes of application.

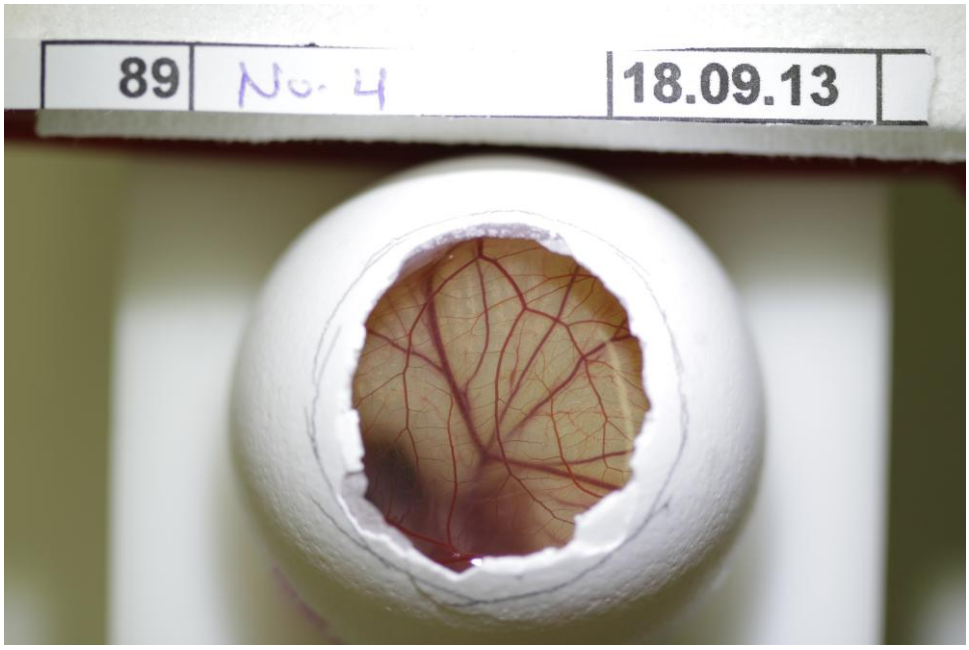

Figure 13: CAM before adding Granulox® spray

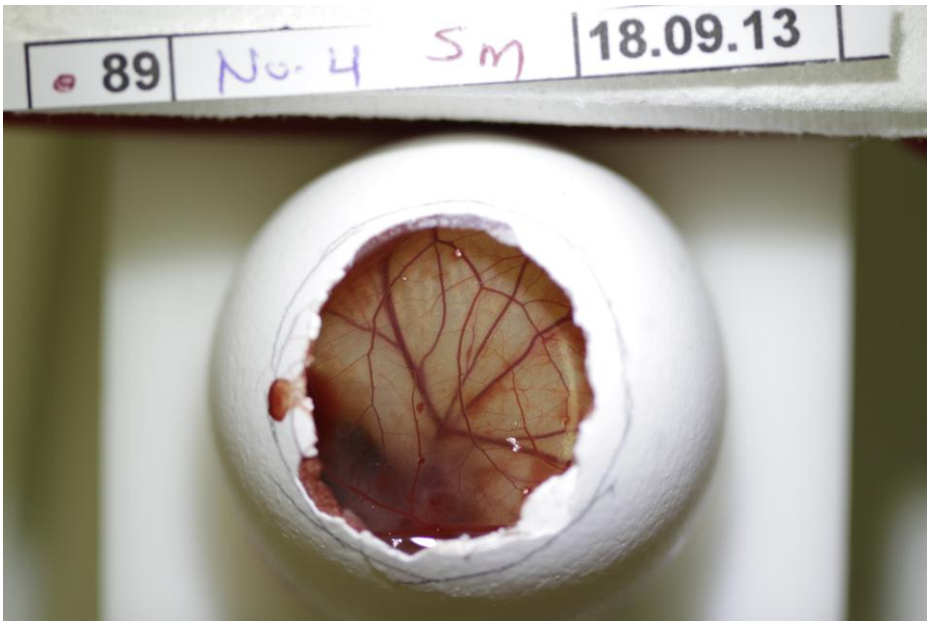

Figure 14: CAM shows no reaction 5 minutes after adding Granulox® spray

Furacin® ointment is a yellow opaque ointment, therefore calculating of IS was impossible. The test was done as follows: The ointment was put on a plastic slide, then the slide was turned on the CAM for 5 minutes and thereafter the slide was removed. Only one egg showed no reaction, 7 eggs showed hemorrhage with severity 1 and one egg showed coagulation with severity 2 (Table 12).

Table 12: Irritation potency of Furacin® ointment after 5 minutes

Day	Egg		IS	After 5 minutes		After 24 hours
				Main reaction	Se-verity	
1	1	Because of the yellow color of the ointment, evaluation of test reactions was only possible after removing the ointment 5 minutes after application	Cannot be calculated	No reaction	0	Died
1	2			Coagulation	2	Died
1	3			Hemorrhage	1	Died
2	4			Hemorrhage	1	Died
2	5			Hemorrhage	1	Died
2	6			Hemorrhage	1	Died
3	7			Hemorrhage	1	Died
3	8			Hemorrhage	1	Died
3	9			Hemorrhage	1	Died
Number of eggs tested: **9**						
Mean of irritation score (IS): **Cannot be calculated**						
Irritation score (IS) range according to Spielmann: **Cannot be known**						
Most common main reaction after 5 minutes: **Hemorrhage**						
Mean of the reaction severity after 5 minutes: **1**						
Percentage of alive eggs after 24 hours: **0%**						

The difference between normal CAM before application of Furacin® ointment (Figure 15) and after application is visible, the CAM shows hemorrhage of severity 1 (Figure 16).

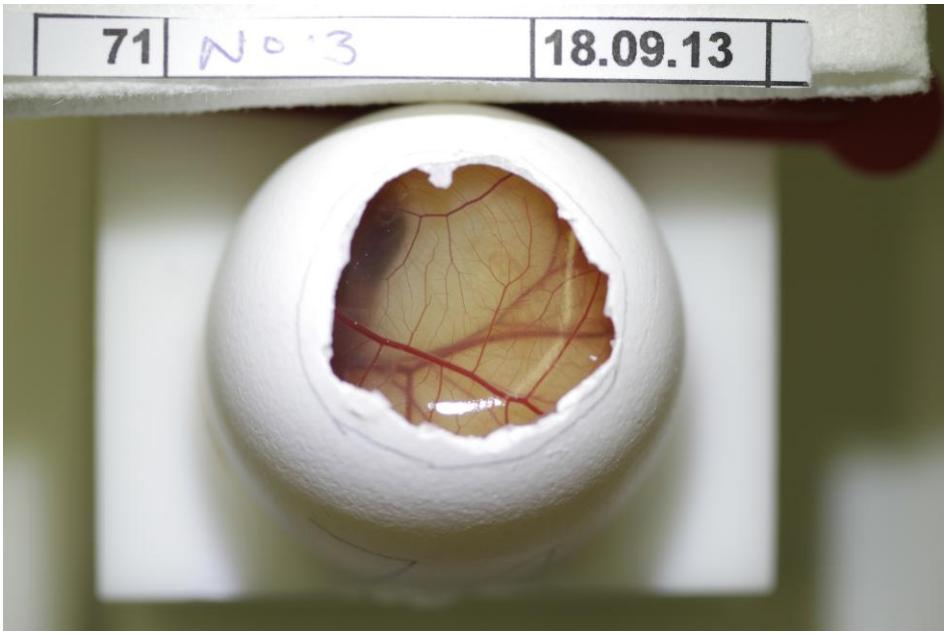

Figure 15: CAM before applying Furacin® ointment

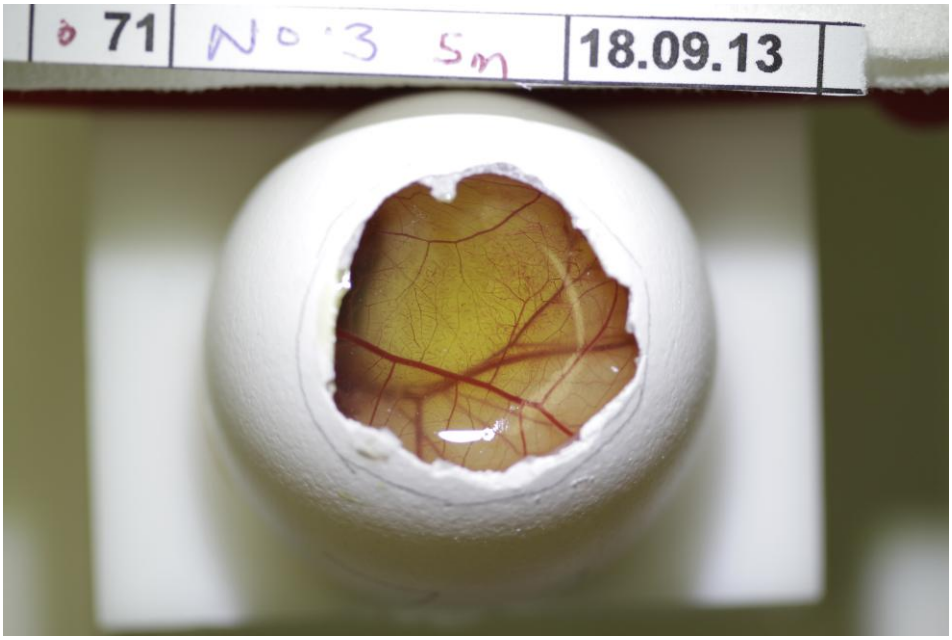

Figure 16: CAM shows hemorrhage 5 minutes after applying Furacin® ointment and removal of the plastic side the ointment

Responding to polihexanide 0.04% in Ringer solution, 2 CAMs reacted with lysis severity 2, the other 7 CAMs showed no reaction. 2 eggs were alive after 24 hours (Table 13).

Table 13: Irritation potency of polihexanide 0.04% in Ringer solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemor-rhage	Lysis	Coagulation		Main reaction	Se-verity	
2	1	No reaction	82	No reaction	5.1	Lysis	2	Died
2	2	No reaction	180	No reaction	2.8	Lysis	2	Died
1	3	No reaction	No reaction	No reaction	0	No reaction	0	Alive
2	4	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	5	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	6	No reaction	No reaction	No reaction	0	No reaction	0	Alive
3	7	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	8	No reaction	No reaction	No reaction	0	No reaction	0	Died
3	9	No reaction	No reaction	No reaction	0	No reaction	0	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **0.9**								
Standard deviation of irritation score (IS): **1.83**								
Irritation score (IS) range according to Spielmann: **No reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **0.22**								
Percentage of alive eggs after 24 hours: **22%**								

Figure 17 shows the CAM before application of polihexanide solution; figure 18 shows it after the application, the CAM shows lysis of severity 2.

Figure 17: CAM before adding polihexanide 0.04% in Ringer solution

Figure 18: CAM shows Lysis 5 minutes after adding polihexanide 0.04% in Ringer solution

Lipofundin is a white non-opaque emulsion that made the monitoring of the CAM and calculating the IS impossible, so the test was done as follows: The solution was put onto the CAM to cover approximately half of its surface (Figure 20), after 5 minutes, the solution carefully was rinsed off using a sterilized tissue to absorb out test material (Figure 21), then the main reaction (hemorrhage, lysis, coagulation) was recorded, and the main reaction was scored (Table 14).

Table 14: Irritation potency of polihexanide 0.05% in Lipofundin® o/w emulsion

Day	Egg		IS	After 5 minutes		After 24 hours
				Main reaction	Se-verity	
4	1	Because of the white color of the emulsion, evaluation of test reactions was only possible after removing the emulsion 5 minutes after application	Cannot be calculated	No reaction	0	Died
4	2			No reaction	0	Died
4	3			No reaction	0	Died
5	4			No reaction	0	Died
5	5			No reaction	0	Died
6	6			No reaction	0	Died
6	7			No reaction	0	Alive
6	8			No reaction	0	Alive
7	9			No reaction	0	Died
Number of eggs tested: **9**						
Mean of the irritation score (IS): **Cannot be calculated**						
Irritation score (IS) range according to Spielmann: **Cannot be known**						
Most common main reaction after 5 minutes: **No reaction**						
Mean of the reaction severity after 5 minutes: **0**						
Percentage of alive eggs after 24 hours: **22%**						

In figure 19, the CAM is shown before the application of polihexanide 0.05% in Lipofundin®. In figure 20 the reaction of the CAM is shown one minute after applying. In figure 21 the CAM shows no reaction 5 minutes after applying the emulsion, hemorrhage was caused by the sterilized tissue which was used to absorb the test material.

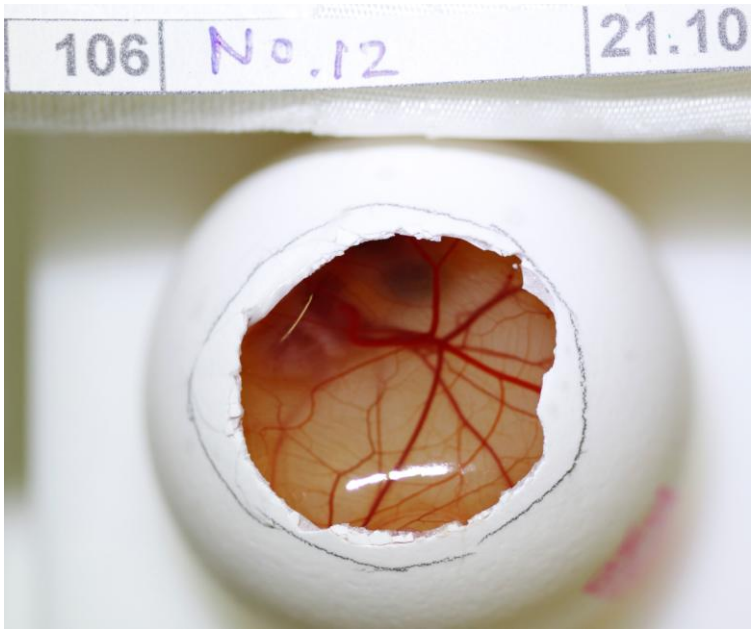

Figure 19: CAM before applying polihexanide 0.05% in Lipofundin®

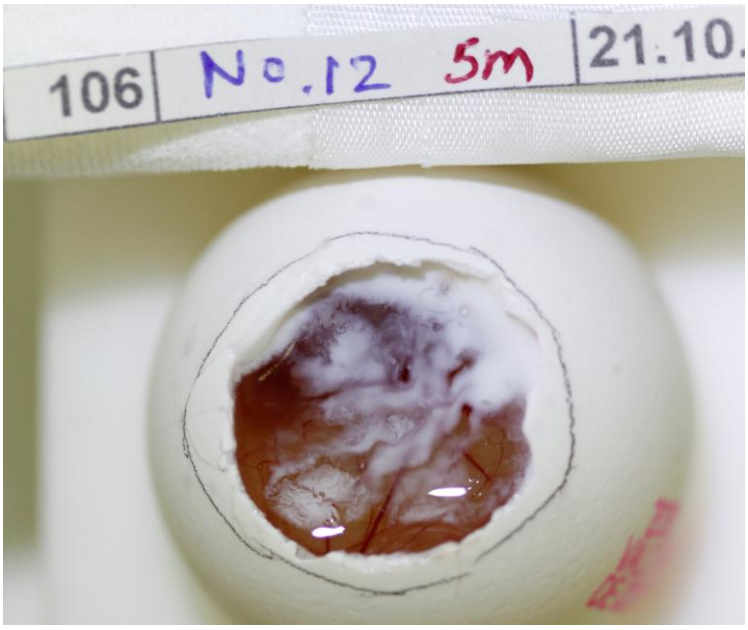

Figure 20: CAM one minute after applying polihexanide 0.05% in Lipofundin®

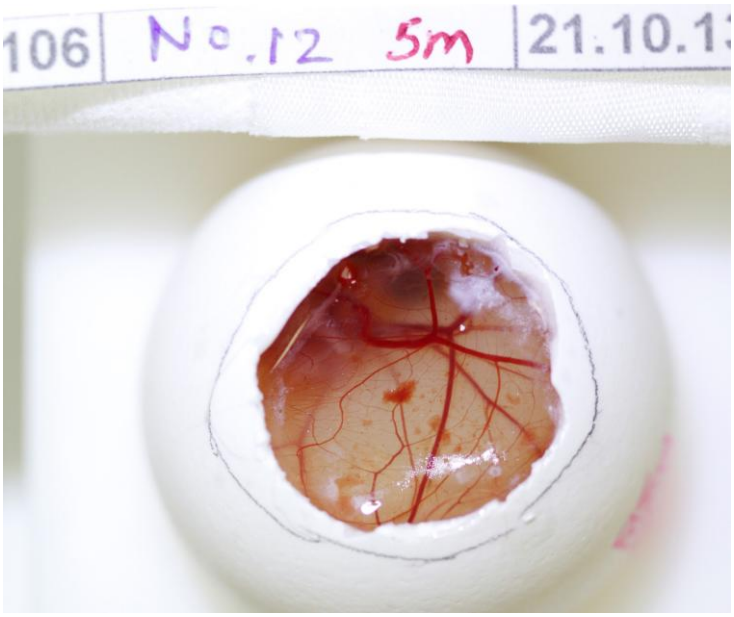

Figure 21: CAM shows no reaction 5 minutes after applying polihexanide 0.05% in Lipofundin®; the hemorrhage was caused by the sterilized tissue which was used to absorb the test material

Braunovidon® is a brown ointment that made the monitoring of CAM and calculating the IS impossible, so the test was done as follows: The ointment was put on a plastic slide, then the slide was turned on the CAM for 5 minutes and then it was removed, thereafter the main reaction (hemorrhage, lysis, coagulation) was recorded and scored. 4 eggs did not show any reaction, 3 showed hemorrhage and 3 showed lysis with severity 1 (Table 15).

Table 15: Irritation potency of Braunovidon® ointment after 5 minutes

Day	Egg		IS	After 5 minutes		After 24 hours
				Main reaction	Se-verity	
4	1	Because of the brown color of the ointment, evaluation of test reactions was only possible after removing the ointment 5 minutes after application	Cannot be calculated	No reaction	0	Died
4	2			Hemorrhage	1	Died
4	3			Hemorrhage	1	Died
5	4			No reaction	0	Died
6	5			Hemorrhage	1	Died
6	6			No reaction	0	Died
6	7			No reaction	0	Died
6	8			Lysis	1	Died
7	9			Lysis	1	Died
Number of eggs tested: **9 eggs**						
Mean of irritation score (IS): **Cannot be calculated**						
Irritation score (IS) range according to Spielmann: **Cannot be known**						
Most common main reaction after 5 minutes: **Hemorrhage**						
Mean of the reaction severity after 5 minutes: **0.55**						
Percentage of alive eggs after 24 hours: **0%**						

Figure 23 shows hemorrhage 5 minutes after applying Braunovidon® ointment. For comparison, the CAM before the application is shown in figure 22.

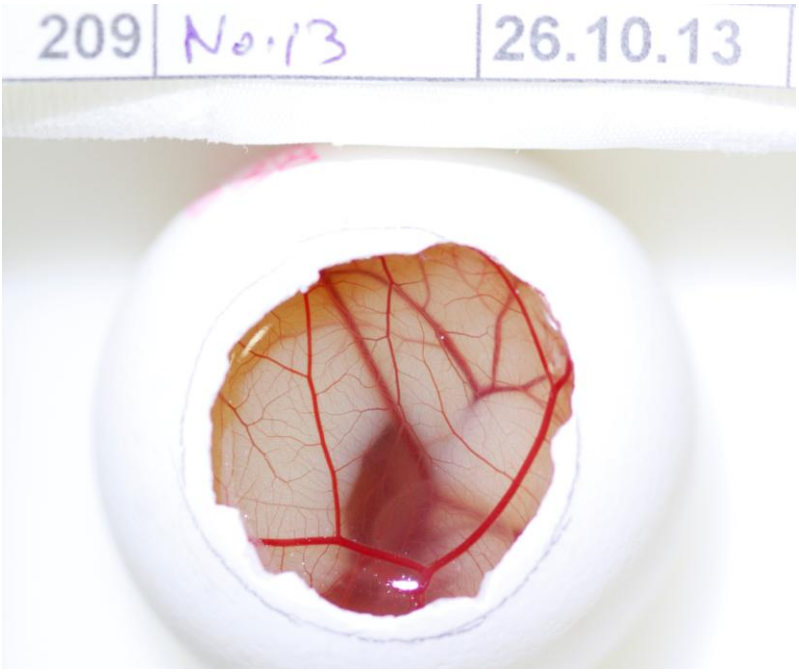

Figure 22: CAM before applying Braunovidon® ointment

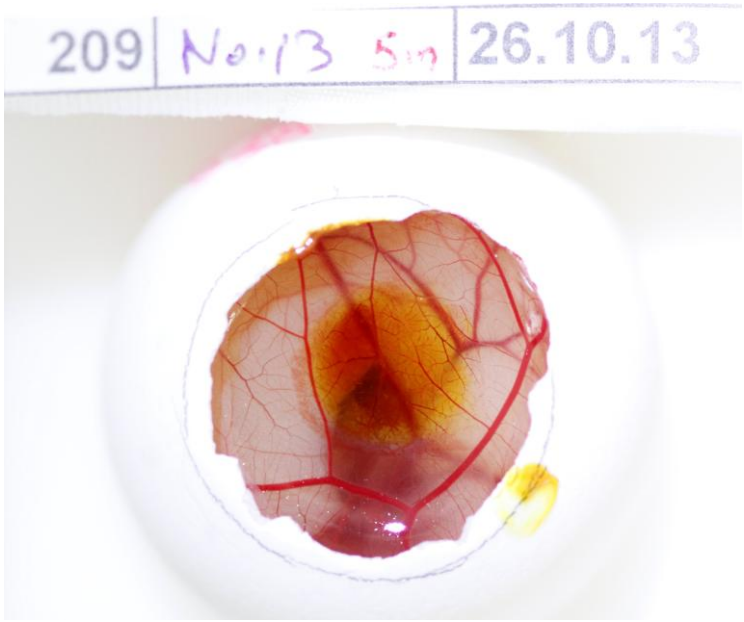

Figure 23: CAM shows hemorrhage 5 minutes after applying Braunovidon® ointment

Flammazine® cream is a white cream that made the monitoring of CAM and calculating the IS impossible, hence the cream was put on a plastic slide, which was turned on the CAM for 5 minutes and then it was removed. Thereafter the main reaction (hemorrhage, lysis, coagulation) was recorded and scored. 8 eggs did not show any reaction while only one showed lysis severity 1 (Table 16).

Table 16: Irritation potency of Flammazine® cream after 5 minutes

Day	**Egg**		**IS**	**After 5 minutes**		**After 24 hours**
				Main reaction	Se-verity	
4	1	Because of the white color of the cream, evaluation of test reactions was only possible after removing the cream 5 minutes after application	Cannot be calculated	No reaction	0	Died
4	2			No reaction	0	Died
4	3			No reaction	0	Died
5	4			No reaction	0	Died
6	5			No reaction	0	Died
6	6			No reaction	0	Died
6	7			No reaction	0	Died
6	8			No reaction	0	Died
7	9			Lysis	1	Died
Number of eggs tested: **9 eggs**						
Mean of irritation score (IS): **Cannot be calculated**						
Irritation score (IS) range according to Spielmann: **Cannot be known**						
Most common main reaction after 5 minutes: **Lysis**						
Mean of the reaction severity after 5 minutes: **0.11**						
Percentage of alive eggs after 24 hours: **0%**						

In figures 24 and 25 the CAM is shown before and after 5 min of Flammazine® application. The CAM was without symptoms of irritation.

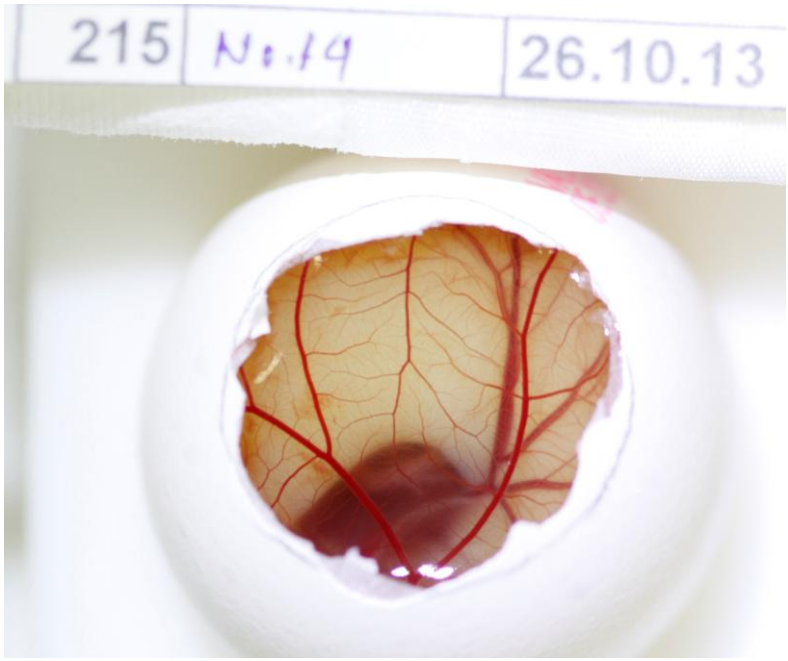

Figure 24: CAM before applying Flammazine® cream

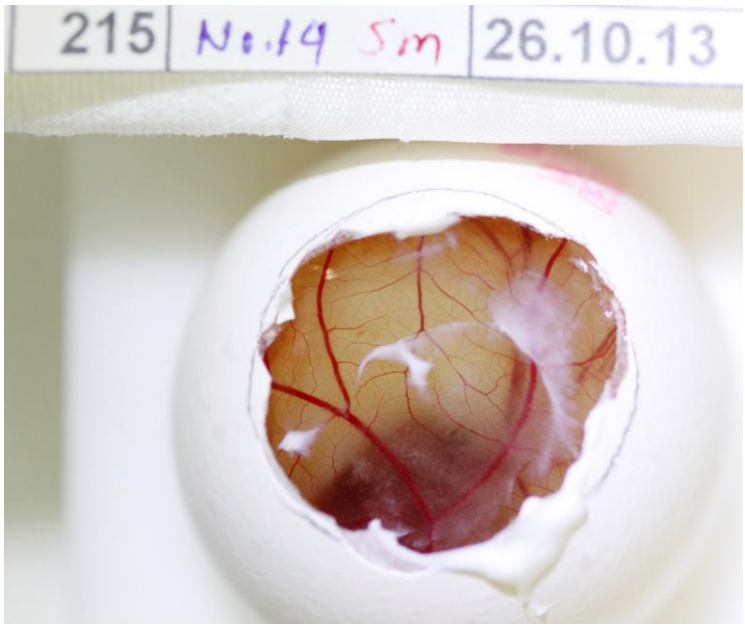

Figure 25: CAM shows no reaction 5 minutes after applying Flammazine® cream

Chlorhexidine digluconate induced severe reactions. The main reaction to be noticed was coagulation (6 eggs); 3 eggs showed lysis. All the eggs died after 24 hours (Table 17).

Table 17: Irritation potency of chlorhexidine digluconate 0.5% solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
4	1	90	214	110	11.2	Coagulation	2	Died
4	2	301	60	77	12.3	Coagulation	2	Died
5	3	301	120	100	10.2	Coagulation	2	Died
5	4	301	70	301	5.3	Lysis	1	Died
5	5	200	301	220	4.1	Coagulation	1	Died
6	6	301	210	301	2.1	Lysis	1	Died
7	7	94	22	301	9.9	Lysis	2	Died
7	8	85	32	120	15.3	Coagulation	2	Died
7	9	80	30	150	14.5	Coagulation	2	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **9.4**								
Standard deviation of irritation score (IS): **4.63**								
Irritation score (IS) range according to Spielmann: **Severe reaction**								
Most common main reaction after 5 minutes: **Coagulation**								
Mean of the reaction severity after 5 minutes: **1.66**								
Percentage of alive eggs after 24 hours: **0%**								

Figure 27 presents lysis after application of 0.5% chlorhexidine digluconate solution. For comparison, one egg from this trial before the application of chlorhexidine is shown (Figure 26).

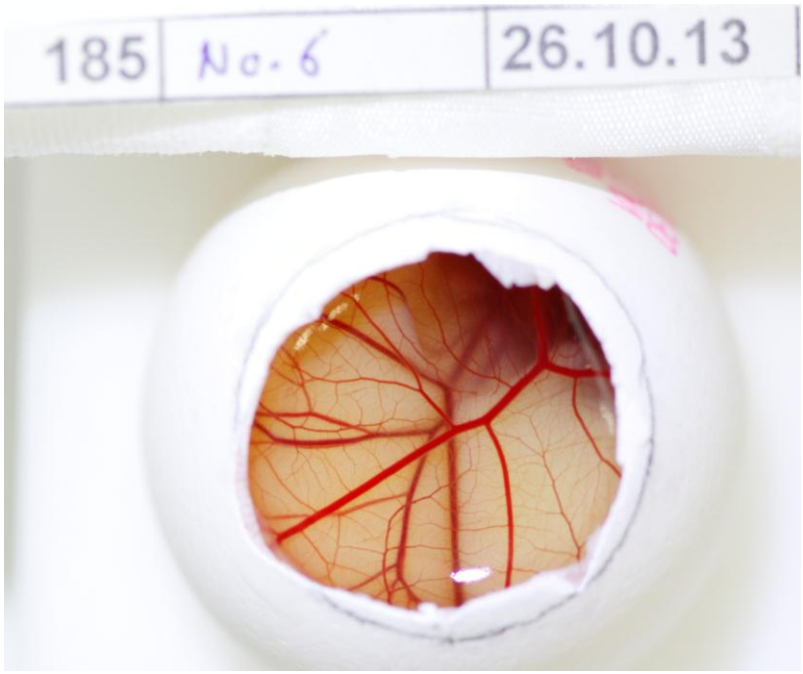

Figure 26: CAM before adding chlorhexidine digluconate 0.5% solution

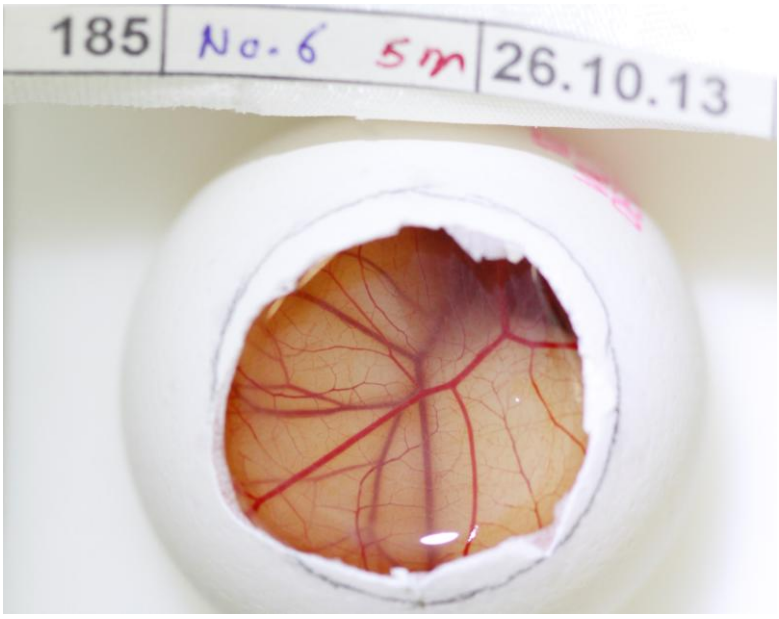

Figure 27: CAM shows lysis 5 minutes after adding chlorhexidine digluconate 0.5% solution

Bepanthen® Cream is a white cream that made the monitoring of CAM and calculating the IS impossible, so the cream was put on a plastic slide which was turned on the CAM for 5 minutes, thereafter the slide was removed and the main reaction (hemorrhage, lysis, coagulation) was recorded and scored. 3 CAMs showed hemorrhage severity 1. The other six did not show any reaction (Table18).

Table 18: Irritation potency for Bepanthen® cream after 5 minutes

Day	Egg		IS	After 5 minutes		After 24 hours
				Main reaction	Se-verity	
4	1	Because of the white color of the cream, evaluation of test reactions was only possible after removing the cream 5 minutes after application	Cannot be calculated	No reaction	0	Died
4	2			No reaction	0	Died
4	3			No reaction	0	Died
5	4			No reaction	0	Died
6	5			Hemorrhage	1	Died
6	6			Hemorrhage	1	Died
6	7			No reaction	0	Died
6	8			Hemorrhage	1	Died
7	9			No reaction	0	Died
Number of eggs tested: **9 eggs**						
Mean of irritation score (IS): **Cannot be calculated**						
Irritation score (IS) range according to Spielmann: **Cannot be known**						
Most common main reaction after 5 minutes: **Hemorrhage**						
Mean of the reaction severity after 5 minutes: **0.33**						
Percentage of alive eggs after 24 hours: **0%**						

In figures 28 and 29 the CAM is shown before and after application of Bepanthen® cream, no reaction was induced in the selected example (Figure 29).

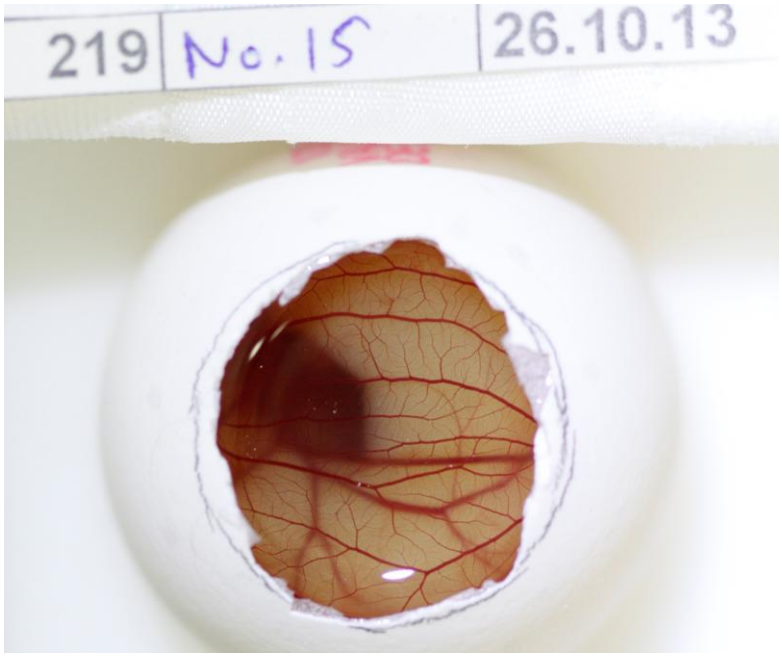

Figure 28: CAM before applying Bepanthen® cream

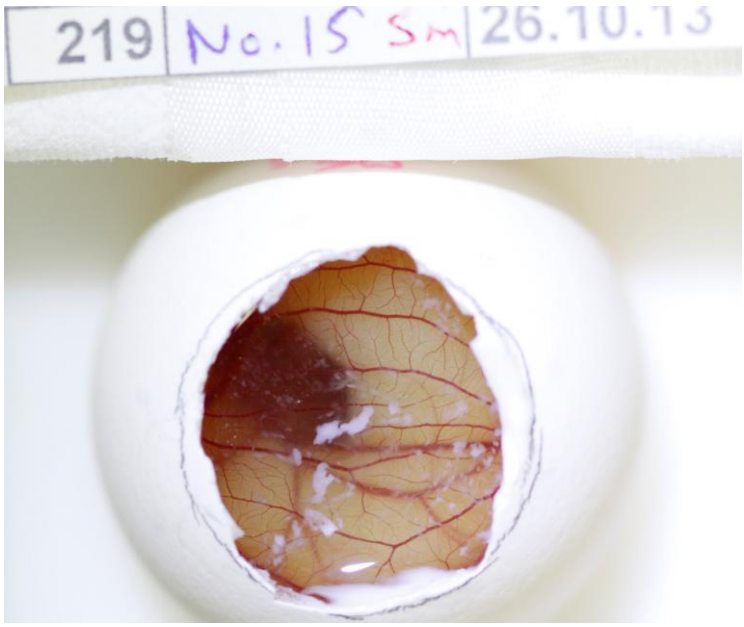

Figure 29: CAM shows no reaction 5 minutes after applying Bepanthen® cream

Dexpanthenol in 5% solution was tolerated without any reaction of the CAM (Table 19). All eggs were died after 24 hours.

Table 19: Irritation potency of dexpanthenol 5% solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemor-rhage	Lysis	Coagulation		Main reaction	Se-verity	
4	1	No reaction	No reaction	No reaction	0	No reaction	0	Died
4	2	No reaction	No reaction	No reaction	0	No reaction	0	Died
4	3	No reaction	No reaction	No reaction	0	No reaction	0	Died
5	4	No reaction	No reaction	No reaction	0	No reaction	0	Died
5	5	No reaction	No reaction	No reaction	0	No reaction	0	Died
6	6	No reaction	No reaction	No reaction	0	No reaction	0	Died
6	7	No reaction	No reaction	No reaction	0	No reaction	0	Died
6	8	No reaction	No reaction	No reaction	0	No reaction	0	Died
7	9	No reaction	No reaction	No reaction	0	No reaction	0	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **0**								
Standard deviation of irritation score (IS): **0**								
Irritation score (IS) range according to Spielmann: **No reaction**								
Most common main reaction after 5 minutes: **No reaction**								
Mean of the reaction severity after 5 minutes: **0**								
Percentage of alive eggs after 24 hours: **0%**								

Between the appearance of CAM before application of 5% dexpanthenol (Figure 30) and thereafter (Figure 31), no difference was recognized.

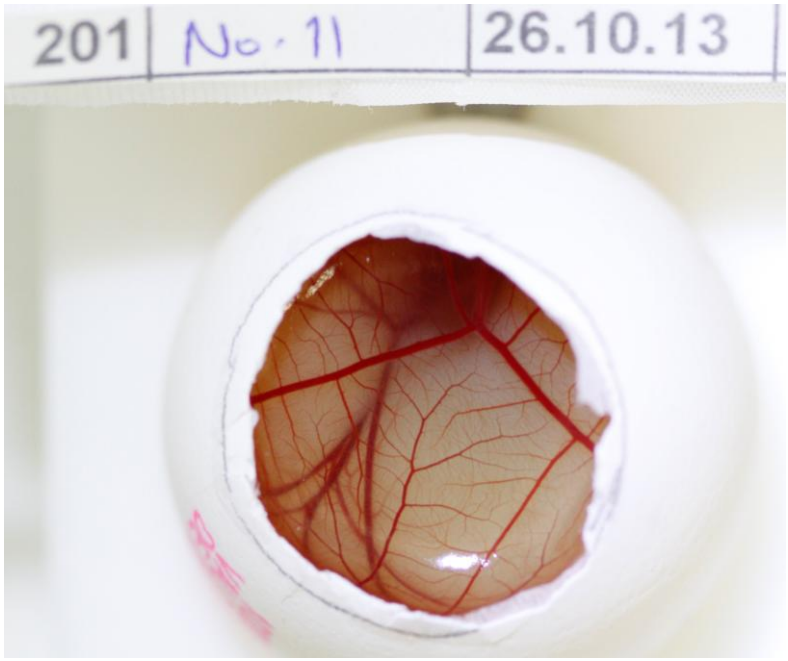

Figure 30: CAM before adding dexpanthenol 5% solution

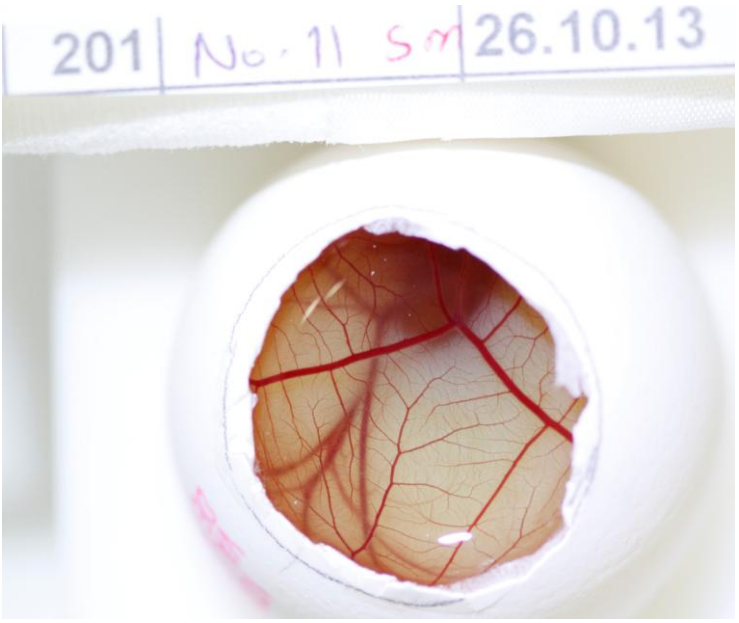

Figure 31: CAM showed no reaction 5 minutes after adding dexpanthenol 5% solution

After application of 0.698% sodium thiocyanate, 4 eggs showed lysis with severity 1, the other 5 eggs did not show any reaction (Table 20). The classification for IS was “slight reaction”.

Table 20: Irritation potency of sodium thiocyanate 0.698% solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemor-rhage	Lysis	Coagulation		Main reaction	Se-verity	
4	1	No reaction	105	No reaction	4.5	Lysis	1	Died
4	2	No reaction	95	No reaction	4.8	Lysis	1	Died
5	3	No reaction	134	No reaction	3.8	Lysis	1	Died
5	4	No reaction	No reaction	No reaction	0	No reaction	0	Died
5	5	No reaction	No reaction	No reaction	0	No reaction	0	Alive
6	6	No reaction	No reaction	No reaction	0	No reaction	0	Alive
7	7	No reaction	No reaction	No reaction	0	No reaction	0	Died
7	8	No reaction	No reaction	No reaction	0	No reaction	0	Died
7	9	No reaction	57	No reaction	5.6	Lysis	1	Died
Number of eggs tested: **9**								
Mean of the irritation score (IS): **2.1**								
Standard deviation of irritation score (IS): **2.51**								
Irritation score (IS) range according to Spielmann: **Slight reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **0.33**								
Percentage of alive eggs after 24 hours: **22%**								

Figure 32 shows the CAM before application of 0.698% sodium thiocyanate, figure 33 shows it thereafter. In figure 33 the severity of lysis was 1.

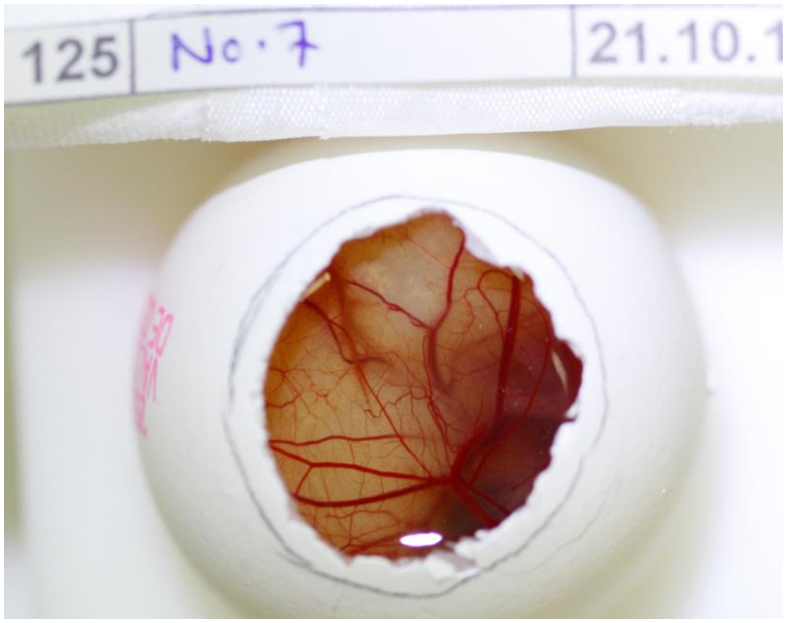

Figure 32: CAM before adding 0.698% sodium thiocyanate solution

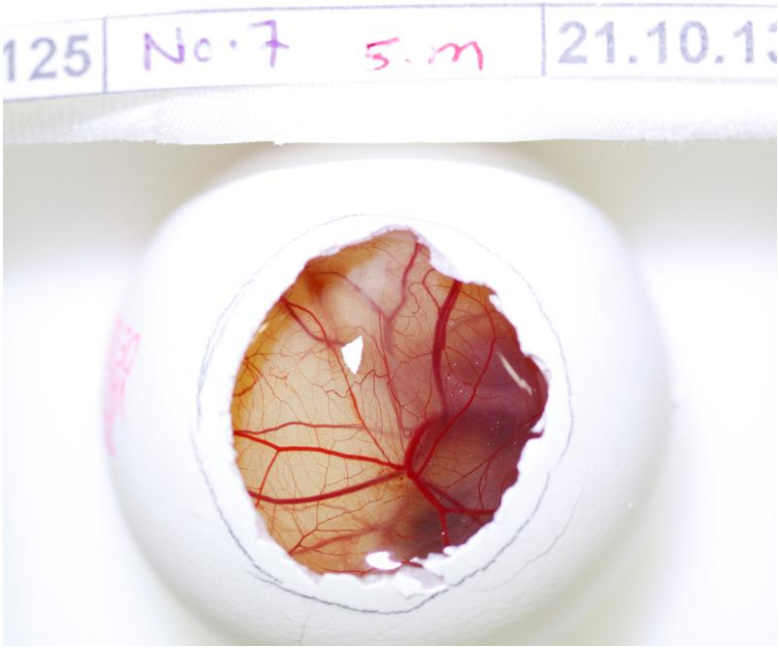

Figure 33: CAM shows lysis 5 minutes after adding 0.698% sodium thiocyanate solution

After application of 0.5% hydrogen peroxide, all 9 eggs showed lysis, the severity ranges between 1 and 3 (Table 21). The time after beginning of lysis ranged between 30 and 155 seconds.

Table 21: Irritation potency of 0.5% hydrogen peroxide

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
4	1	No reaction	30	No reaction	6.3	Lysis	2	Died
4	2	No reaction	55	No reaction	5.7	Lysis	1	Died
4	3	No reaction	120	No reaction	4.2	Lysis	2	Died
5	4	No reaction	155	No reaction	3.4	Lysis	1	Died
5	5	No reaction	54	No reaction	5.7	Lysis	2	Died
6	6	No reaction	31	No reaction	6.3	Lysis	3	Died
6	7	No reaction	50	No reaction	5.8	Lysis	3	Died
6	8	No reaction	47	No reaction	5.9	Lysis	3	Died
7	9	No reaction	30	No reaction	6.3	Lysis	3	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **5.5**								
Standard deviation of irritation score (IS): **1.02**								
Irritation score (IS) range according to Spielmann: **Moderate reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **2.2**								
Percentage of alive eggs after 24 hours: **0%**								

In seven eggs, 30-60 seconds after adding 0.5% hydrogen peroxide, bubbles appeared on the surface of the CAM (Figure 35). Figure 34 shows the CAM before adding the solution.

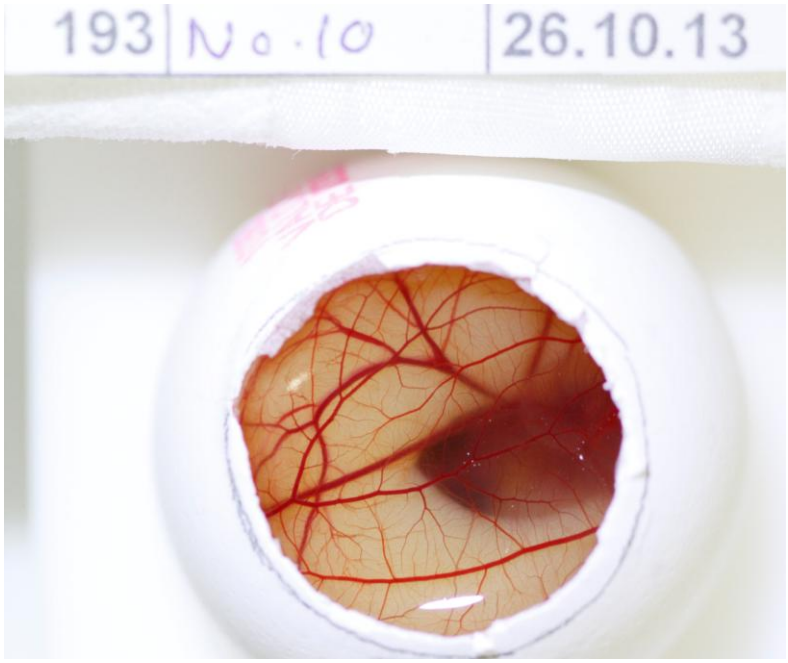

Figure 34: CAM before adding 0.5% hydrogen peroxide

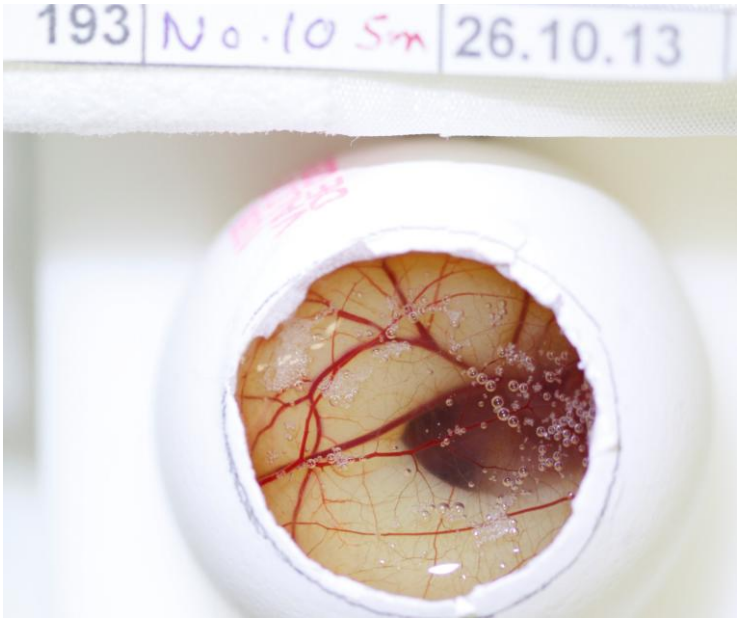

Figure 35: CAM shows lysis and bubbles 5 minutes after adding 0.5% hydrogen peroxide

In the nine eggs, 10 seconds after adding 1.5% hydrogen peroxide solution, bubbles appeared on the surface of the CAM. After 5 minutes, all CAMs developed lysis of severity 2 or 3 (Table 22). After 24 hours, all eggs died.

Table 22: Irritation potency of 1.5% hydrogen peroxide solution

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemorrhage	Lysis	Coagulation		Main reaction	Se-verity	
4	1	No reaction	89	No reaction	4.9	Lysis	3	Died
4	2	No reaction	10	No reaction	6.7	Lysis	3	Died
5	3	No reaction	50	No reaction	5.8	Lysis	3	Died
5	4	No reaction	28	No reaction	6.3	Lysis	3	Died
5	5	No reaction	60	No reaction	5.6	Lysis	2	Died
6	6	No reaction	27	No reaction	6.3	Lysis	2	Died
6	7	No reaction	37	No reaction	6.1	Lysis	3	Died
7	8	No reaction	22	No reaction	6.5	Lysis	3	Died
7	9	No reaction	22	No reaction	6.5	Lysis	3	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **6.1**								
Standard deviation of irritation score (IS): **0.56**								
Irritation score (IS) range according to Spielmann: **Moderate reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **2.77**								
Percentage of alive eggs after 24 hours: **0%**								

While the CAM of all eggs was normal before the application of hydrogen peroxide 1.5% (Figure 36), about 10 seconds later bubbles started to appear on the surface (Figure 37).

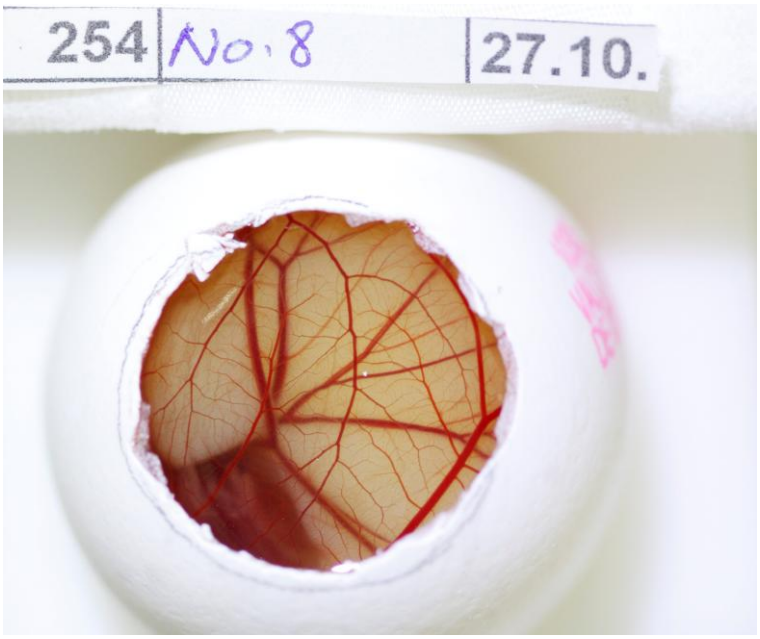

Figure 36: CAM before adding 1.5% hydrogen peroxide

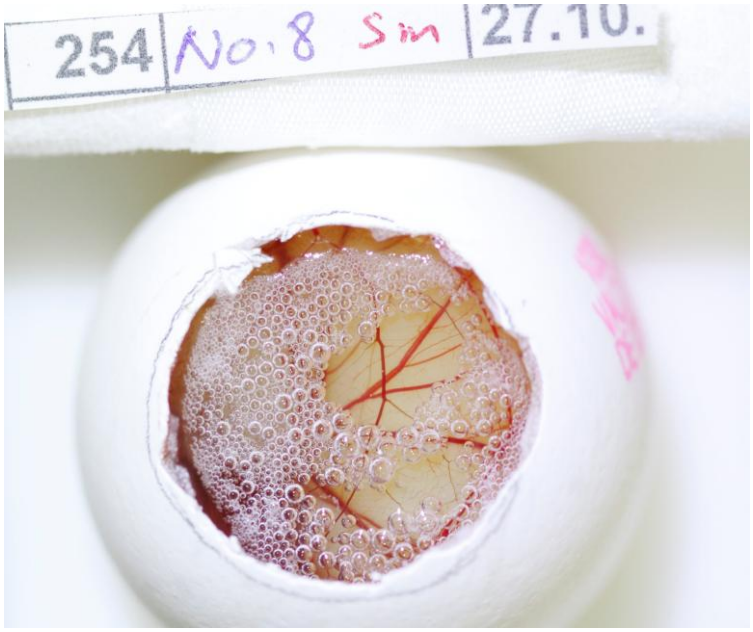

Figure 37: CAM shows lysis 5 minutes after adding 1.5% hydrogen peroxide, bubbles started to appear after 10 seconds of adding

After 5 minutes of applying 1.5% hydrogen peroxide and 0.698% sodium thiocyanate, one egg was without any reaction, 5 eggs showed lysis up to severity 3, 2 eggs showed coagulation (Table 23).

Table 23: Irritation potency of the combination of 1.5% hydrogen peroxide and 0.698% sodium thiocyanate

Day	Egg	Time (s) for reaction to occur			IS	After 5 minutes		After 24 hours
		Hemor-rhage	Lysis	Coagulation		Main reaction	Se-verity	
5	1	No reaction	250	No reaction	1.1	Lysis	1	Died
5	2	No reaction	No reaction	No reaction	0	No reaction	0	Died
5	3	No reaction	No reaction	300	0.03	Coagulation	1	Died
5	4	No reaction	No reaction	255	1.3	Coagulation	1	Died
5	5	No reaction	No reaction	No reaction	0	No reaction	0	Died
6	6	No reaction	106	No reaction	4.5	Lysis	1	Died
6	7	No reaction	42	No reaction	6	Lysis	1	Died
7	8	No reaction	28	No reaction	6.3	Lysis	2	Died
7	9	No reaction	130	No reaction	3.9	Lysis	3	Died
Number of eggs tested: **9**								
Mean of irritation score (IS): **2.6**								
Standard deviation of irritation score (IS): **2.61**								
Irritation score (IS) range according to Spielmann: **Slight reaction**								
Most common main reaction after 5 minutes: **Lysis**								
Mean of the reaction severity after 5 minutes: **1.1**								
Percentage of alive eggs after 24 hours: **0%**								

In seven eggs, 10 seconds after adding the combination of hydrogen peroxide 1.5% and sodium thiocyanate 0.698%, the vessels of the CAM became black in color (Figure 39). In one egg, 10 seconds after adding the mixture, bubbles appeared on the surface of the CAM. For comparison, the CAM is seen before application (Figure 38).

Figure 38: CAM before adding H_2O_2 1.5% solution in combination with sodium thiocyanate 0.698%

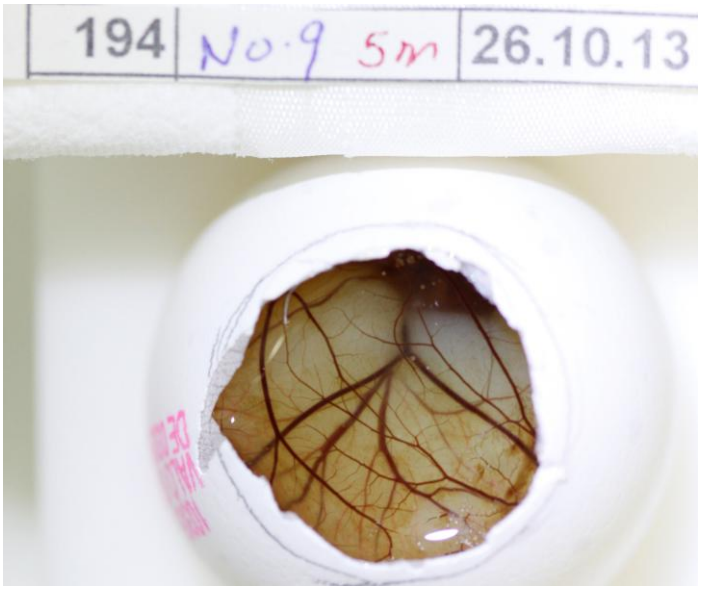

Figure 39: CAM shows lysis and vessels of the CAM became black in color after adding H_2O_2 1.5% solution in combination with sodium thiocyanate 0.698%

4.2 Statistic evaluation of the irritation scores

The results of descriptive statistics are summarized in table 24. The mean IS for the positive controls were 10.0 ± 2.39 for 1% SDS and 15.3 ± 2.10 for 0.1 N NaOH. The mean for the negative control was 0. After applying test substances, the highest means of IS were found for Octenilin® (10.2 ± 3.33) and 0.5% Chlorhexidine digluconate (9.4 ± 4.63). The IS decreased when the following test substances were used: 0.05% Octenidin + 1.34% dexpanthenol + 0.2% allantoin (8.7 ± 2.3), 1.5% H_2O_2 (6.1 ± 0.56), 0.5% H_2O_2 (5.5 ± 1.02), 1.5% H_2O_2 + 0.698% NaSCN (2.6 ± 2.61), 0.698% NaSCN (2.1 ± 2.51), Dermacyn® (1.1 ± 1.54), 0.004% polihexanide in Ringer solution (0.9 ± 1.83), 5% dexpanthenol (0 ± 0), and Granulox® spray (0 ± 0).

The IS could not be calculated for the other five test substances, Polihexanide 0.05% in Lipofundin®, Flammazine® cream, Bepanthen® cream, Braunovidon® ointment and Furacin® ointment. Therefore, no statistical analysis was estimated with these data.

Table 24: Overview of the test results

Substance	No of eggs	Min	25th Percentile	Median	75th Percentile	Max	Mean	Std. Deviation	IS range	after 5 minutes		Percentage of alive eggs after 24 hours (%)
										Most common main reaction	Mean of the reaction severity	
Controls												
0.9% NaCl solution in dist. water	18	0.0	0.00	0.0	0.00	0.0	0.0	0.00	No reaction	No reaction	0.00	44
1% SDS (positive standard)	18	4.8	8.53	9.9	10.85	15.0	10.0	2.39	Severe reaction	Lysis	1.64	0
0.1 n NaOH (positive standard)	18	8.2	14.55	15.6	16.50	17.5	15.3	2.10	Severe reaction	Hemorrhage	3.00	0
Test substance in which IS can be calculated												
Granulox® spray	9	0.0	0.00	0.0	0.00	0.0	0.0	0.00	No reaction	No reaction	0.00	0
5% Dexpanthenol	9	0.0	0.00	0.0	0.00	0.0	0.0	0.00	No reaction	No reaction	0.00	0
0.04% Polihexanide in Ringer solution	9	0.0	0.00	0.0	1.40	5.1	0.9	1.83	No reaction	Lysis	0.22	22
Dermacyn® wound care	17	0.0	0.00	0.3	2.05	4.9	1.1	1.54	Slight reaction	Lysis	1.00	59
0.698% NaSCN	9	0.0	0.00	0.0	4.65	5.6	2.1	2.51	Slight reaction	Lysis	0.33	22
1.5% H_2O_2+ 0.698% NaSCN	9	0.0	0.02	1.3	5.25	6.3	2.6	2.61	Slight reaction	Lysis	1.10	0
0.5% H_2O_2	9	3.4	4.95	5.8	6.30	6.3	5.5	1.02	Moderate reaction	Lysis	2.20	0
1.5% H_2O_2	9	4.9	5.70	6.3	6.50	6.7	6.1	0.56	Moderate reaction	Lysis	2.77	0
0.05% OCT + 1.34% dexpanthenol + 0.2% allantoin	9	5.5	6.05	9.4	10.05	12.3	8.7	2.30	Moderate reaction	Lysis	1.66	0
0.5% Chlorhexidine digluconate	9	2.1	4.70	10.2	13.40	15.3	9.4	4.63	Severe reaction	Coagulation	1.66	0
Octenilin® wound irrigation solution	9	4.7	7.60	10.0	12.90	15.5	10.2	3.33	Severe reaction	Coagulation	1.62	0

As shown in figure 40, the IS using the positive control 0.1 n NaOH was significant higher compared to the IS of test substances and the other positive control 1% SDS (P<0.0001). The score after 1% SDS application was significant higher compared to 1.5% and 0.5% H_2O_2, 1.5% H_2O_2 + 0.698% NaSCN, 0.698% NaSCN, Dermacyn®, 0.004% polihexanide in Ringer solution, 5% dexpanthenol, and Granulox® spray. In contrast, the IS of Octenilin®, 0.5% chlorhexidine digluconate and 0.05% octenidine+ 1.34% dexpanthenol + 0.2% allantoin were not significant different from the IS of 1% SDS.

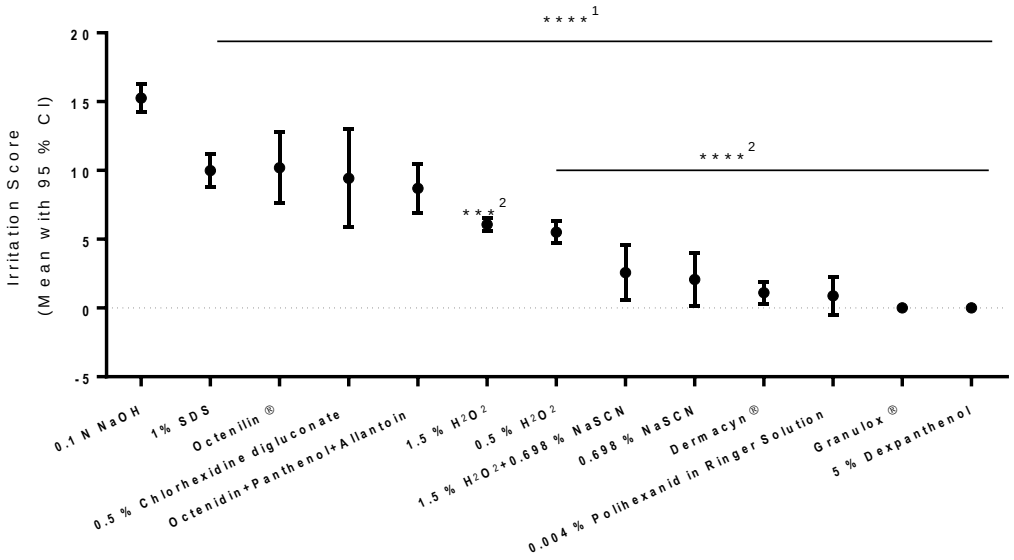

Figure 40: Irritation Scores of test substances compared to positive controls

1**** P<0.0001 compared to 0.1 N NaOH; 2**** P=0.0005 und ****P<0.0001 compared to 1% SDS

While the statistical analysis of the IS for the tested creams could not be calculated, for the other test substances differences were partly significant (Table 25). The formulations on basis of hypochlorite and polihexanide as well as the hemoglobin spray were significantly better tolerable than the octenidine and chlorhexidine based formulations and hydrogen peroxide in both tested concentrations. While 0.04% polihexanide in Ringer solution was significantly more tolerable than 1.5% H_2O_2, the difference was negated via the addition of 0.698% NaSCN to the H_2O_2 (p>0.999). By adding dexpanthenol and allantoin, the irritant of effect of octenidine was decreased slightly (p 0.279). Dermacyn, 0.04% polihexanide in Ringer solution and Granulox did not significantly differ in irritative effect (each p>0.99). The IS of Octenilin® and 0.5% chlorhexidine digluconate also did not vary significantly (p>0.999).

Table 25: Significant differences between test substances

Comparison	Mean Diff.	95% CI of diff.	Sum-mary	Adjusted P Value
Significant differences between test substances				
Octenilin® vs. Dermacyn®	9.082	5.958 to 12.21	****	< 0.0001
Octenilin® vs. Granulox®	10.2	6.627 to 13.77	****	< 0.0001
Octenilin® vs. 0.004% polihexanide in Ringer Solution	9.322	5.750 to 12.89	****	< 0.0001
Octenilin® vs. 5% dexpanthenol	10.2	6.627 to 13.77	****	< 0.0001
Octenilin® vs. 0.698% NaSCN	8.122	4.550 to 11.69	****	< 0.0001
Octenilin® vs. 0.5% H_2O_2	4.689	1.116 to 8.262	**	0.0014
Octenilin® vs. 1.5% H_2O_2	4.122	0.5495 to 7.695	**	0.0096
Octenilin® vs. 1.5% H_2O_2+0.698% NaSCN	7.63	4.057 to 11.20	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. Dermacyn®	7.571	4.447 to 10.70	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. Granulox®	8.689	5.116 to 12.26	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. 0.004% polihexanide in Ringer Solution	7.811	4.238 to 11.38	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. 5% dexpanthenol	8.689	5.116 to 12.26	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. 0.698% NaSCN	6.611	3.038 to 10.18	****	< 0.0001
Octenidin + dexpanthenol + allantoin vs. 1.5% H_2O_2 + 0.698% NaSCN	6.119	2.546 to 9.692	****	< 0.0001
Dermacyn® vs. 0.5% chlorhexidine digluconate	-8.316	-11.44 to -5.191	****	< 0.0001
Dermacyn® vs. 0.5% H_2O_2	-4.393	-7.518 to -1.269	***	0.0004
Dermacyn® vs. 1.5% H_2O_2	-4.96	-8.084 to -1.836	****	< 0.0001
Granulox® vs. 0.5% chlorhexidine digluconate	-9.433	-13.01 to -5.861	****	< 0.0001
Granulox® vs. 0.5% H_2O_2	-5.511	-9.084 to -1.938	****	< 0.0001
Granulox® vs. 1.5% H_2O_2	-6.078	-9.650 to -2.505	****	< 0.0001
0.004 % Polihexanide in Ringer solution vs. 0.5% chlorhexidine digluconate	-8.556	-12.13 to -4.983	****	< 0.0001
0.004% Polihexanide in Ringer solution vs. 0.5% H_2O_2	-4.633	-8.206 to -1.061	**	0.0017
0.004% Polihexanide in Ringer Solution vs. 1.5% H_2O_2	-5.2	-8.773 to -1.627	***	0.0002
0.5% Chlorhexidine digluconate vs. 5% dexpanthenol	9.433	5.861 to 13.01	****	< 0.0001
0.5% Chlorhexidine digluconate vs. 0.698% NaSCN	7.356	3.783 to 10.93	****	< 0.0001
0.5% Chlorhexidine digluconate vs. 0.5% H_2O_2	3.922	0.3495 to 7.495	*	0.0181
0.5% Chlorhexidine digluconate vs. 1.5% H_2O_2 + 0.698% NaSCN	6.863	3.291 to 10.44	****	< 0.0001
5% Dexpanthenol vs. 0.5% H_2O_2	-5.511	-9.084 to -1.938	****	< 0.0001
5% Dexpanthenol vs. 1.5% H_2O_2	-6.078	-9.650 to -2.505	****	< 0.0001
0.698% NaSCN vs. 1.5% H_2O_2	-4	-7.573 to -0.4273	*	0.0142

4.3 Verification of microbial contamination

After finishing the second round of the experiment, it was noticed that most of the eggs died after 24h even though incubation, so a contamination test was done to clarify if there is a relation between contamination and death of the embryos. According to the contamination test, no relation between the contamination of the CAM after 24 hours and the death of the eggs was found (Table 26). Figures 41 and 42 show positive results.

Table 26: Results of the contamination test

Egg number	Test substance	After 24 hours	Findings of contamination
156	0.9% NaCl	Alive	no contamination
143	0.9% NaCl	Died	no contamination
162	Sodium thiocyanate 0.698%	Alive	contamination, more than 50 cfu
137	Sodium thiocyanate 0.698%	Died	contamination, more than 150 cfu
136	Hydrogen peroxide (H_2O_2) 1.5% solution	Died	no contamination
139	Chlorhexidine 0.5% solution	Died	no contamination

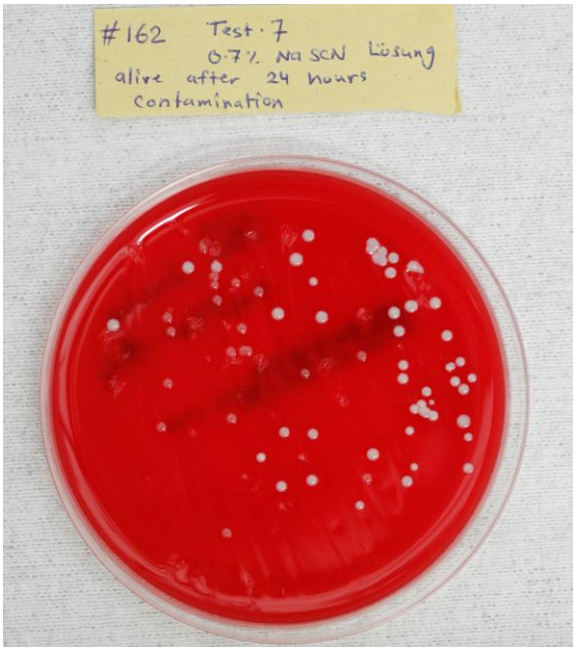

Figure 41: More than 50 colony forming units on blood agar from inoculation of CAM after application of sodium thiocyanate 0.698%, alive after 24 hours

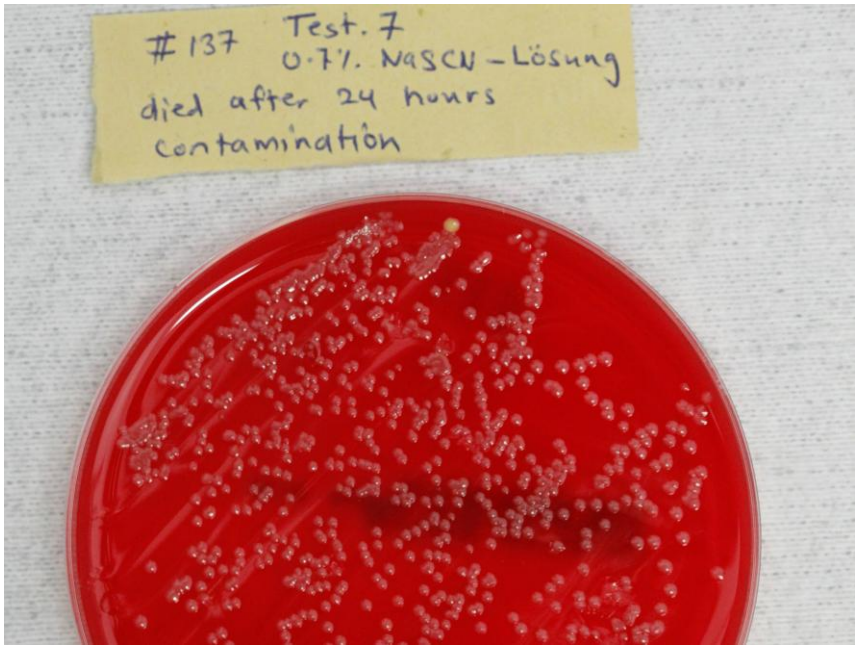

Figure 42: More than 150 colony forming units on blood agar from inoculation of CAM after application of sodium thiocyanate 0.698%, died after 24 hours

5 Discussion

5.1 Method

The CAM is produced as an extraembryonic membrane on the 4th-6th embryo development day (Fuchs and Lindenbaum 1988) as a mesodermal double layer in the chicken egg (Bellairs and Osmond 1998). In this double membrane, a capillary system develops which is connected to the embryo via arteries and veins (Rizzo et al. 1995). The CAM is mainly used for the gas exchange (Rizzo and DeFouw 1993), but also for the calcium supply of the embryo from the yolk sac (Bellairs and Osmond 1998, DeFouw et al. 1989, Schueller 2005). In addition, the CAM is a pool for nitrogen-containing final degradation products (Bellairs and Osmond 1998). The testing on the CAM is not classified as an animal test, since no nerve tissue develops in the chicken egg until the 11th day, so that no pain perception occurs (Liebsch and Spielmann 2002). On the 10th day of incubation, a dense capillary network overlays the area of the CAM which is adjacent to the air bubble. It allows for a time-dependent assessment of the occurrence of hemorrhage, vascular lysis and coagulation in the application area.

The crucial advantage of the HET-CAM assay is the replacement of the Draize Rabbit Eye Test (Spielmann et al. 1991, 1993, Spielmann and Liebsch 1991). The HET-CAM offers easy feasibility, sensitivity and low cost. A disadvantage is the subjective judgment; this is to be countered by a defined assessment scheme.

As the tolerability to the eye is similar to that of wounds (Kramer et al. 2004, Daeschlein et al. 2007, Leaper et al. 2010, Assadian and Kramer 2012, Kramer et al. 2013), more recently the HET-CAM is also recommended as a screening test for the evaluation of wound healing (Kramer et al. 2013). In case of tolerability of an antiseptic agent in the HET-CAM, it can be used on the eye and also on the wound with high probability. This is further confirmed by the fact that the tolerability of cold atmospheric pressure plasma (CAPP) in HET-CAM (Bender et al. 2010, 2011) was initially confirmed by the enucleated eye of slaughter pigs (Hamman et al. 2010). After confirmation of the tolerability of CAPP on the porcine ear (Lademann et al. 2009, 2010) and with pet species (Bender et al. 2013, Bender and Kramer 2016), the prerequisites for the wound treatment of CAPP in humans were introduced. In accordance, after therapy try with CAPP, no severe adverse effects (SAEs) were observed in any case (Isbary et al.2010, 2012, Heinlin et al. 2013, Brehmer et al. 2015, Ulrich et al. 2015). The hypothesis that the tolerability of antiseptic agents on the CAM can be used to predict the tolerability of them on wounds is confirmed by the above findings.

This was the basis for the comparative assessment of selected wound antiseptics on the CAM in order to

- predict the tolerability of the antiseptics on wounds
- compare antiseptics with each other based on their tolerability
- draw conclusions about the suitability of the HET-CAM as a test model.

For further studies using the HET-CAM, the following recommendations can be obtained from the results:

- Increasing the number of eggs tested to be 6-9 for each substance
- monitoring the eggs after 24 hours (physicians need recommendations about the less cytotoxic antiseptics in long term use).

5.2 Results

As expected, the negative control caused no irritation (Table 1). The positive controls fulfill the requirements. 1% SDS solution should give an IS of 10 ± 2, 0.1 n NaOH and IS of 15 ± 3. 1% SDS should show hemorrhage and lysis within the first minute, whereas 0.1 n NaOH shows all 3 phenomena, first hemorrhage within several seconds, later coagulation and lysis at about 1 minute (Spielmann and Liebsch 1991).

Obvious differences in the tolerability between the commercial available wound antiseptics were noticed (Figure 43). In the figure, the IS arranged in ascending order.

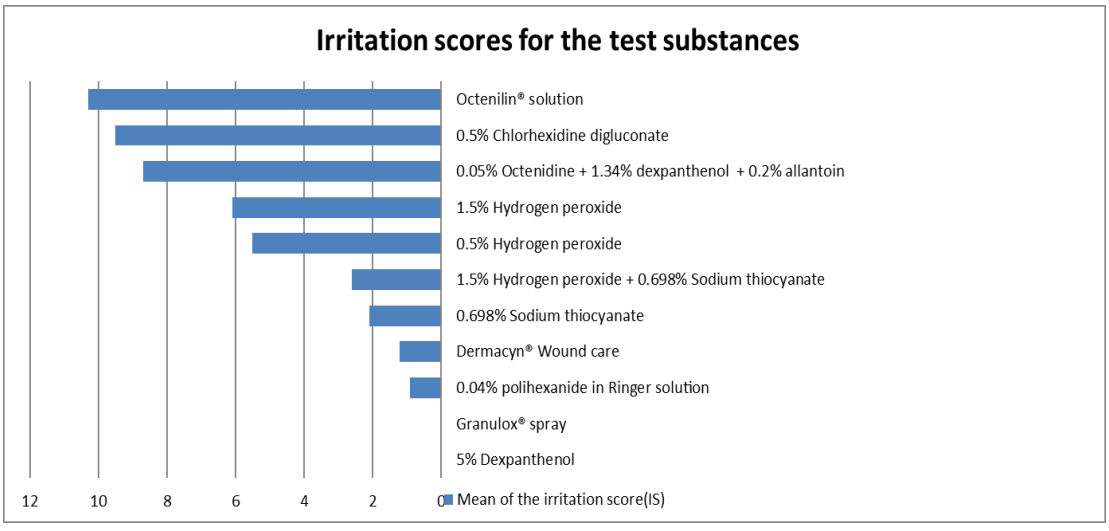

Figure 43: Irritation scores (IS) arranged in ascending order

The wound treatment agent Granulox® spray may have a low antiseptic effect due to the content of 0.7% phenoxyethanol; but yet this has not been investigated. The principle is that the hemoglobin contained in Granulox®, which is purified from porcine blood, transports oxygen from the environment to the wound base and thereby improves wound healing. Until the publication of Strohal et al. (2016), a wound healing enhancement was demonstrated in 37 clinical trials. However, since the spray can be combined with antiseptics such as polihexanide, octenidine and PVP-iodine, the last two have to be thoroughly rinsed (Strohal et al. 2016). The finding of the lack of irritation of Granulox® is of practical relevance for further considerations to combine it with antiseptics.

For polihexanide 0.04% in Ringer solution the IS was 0.22, for 0.05% polihexanide in Lipofundin® no irritation was induced, both receive the classification "no reaction". Analogously Lavanid with the same content of 0.04% polihexanide was tolerated without any irritation (Marquardt et al. 2010). This corresponds to the tolerability observed during clinical application (Hübner and Kramer 2010, Kramer 2016, Kramer et al. 2017). Regarding re-epithelization, 0.04% of polihexanide was significantly superior to PVP-iodine 10% and silver nitrate 1%. In contrast to PVP-iodine and silver nitrate, no deep necrosis and no fibrin deposits were induced by polihexanide (Daeschlein et al. 2007). In comparison to chlorhexidine, the re-epithelization of polihexanide was significantly faster and less painful (Muangman et al. 2011). In combination with surface active betaine the irritation action of polihexanide is increased to “slight reaction”. The same irritation response was reported by Marquardt et al. (2010). Deu to the combination, the antimicrobial effect is enhanced (Müller et al. 2007, López-Rojasa et al. 2017), the in vitro cytotoxicity is reduced (Müller et al. 2007) and cleaning performance is improved (Kaehn 2009). The results underline that polihexanide in combination with polyethylenglycole is to be preferred for repeated application with therapeutic goal, whereas the combination with the betaine is suitable for one-time cleansing of dirty contaminated wounds (Kramer et al. 2017). In comparison to 0.89% NaCl solution, polihexanide with 2 weeks follow-up period, gave more rapid bacterial elimination, faster wound healing, less pain, less exudates and increased granulation (Valenzuela and Perucho 2008). In a multi centric study, polihexanide in combination with betaine, gave a promotion of wound healing (Durante et al. 2014).

Dermacyn® wound care follows polihexanide with IS of 1.2 with Irritation score range "slight" (Table 24), but the difference was not significant. No wound healing inhibition is to be expected clinically, it is even expected that the wound healing will be with low risk of inflammation (Kramer et al. 2017). After the successful stabilization of the combination of NaOCl/HOCl, an ecological new development has been developed, since aqueous

NaCl solution is electrochemically converted to NAOCl/HOCl (the activated solution is also referred as super oxidized water) (Thorn et al. 2012). In agreement with the HET-CAM, there was no evidence for cytotoxicity in the 3D skin model (D'Atanasio et al. 2015). It should be emphasized that bacterial cells can be killed but human cells survive, only by polihexanide (Wiegand et al. 2009) and NaOCl (Crabtree et al. 2001) in co-culture of bacteria and human cells. This underlines the therapeutic range of both drugs. Amazingly, increased number of clinical studies with convincing results on hypochlorite wound antiseptics allow the conclusion that hypochlorite based antiseptics are the antiseptics of choice of intensive antiseptic, cleaning of contaminated traumatic wounds and for repeated antiseptic treatment of chronic wounds until the end of the cleaning phase of the wound (Kramer 2016).

In both tested concentrations of 1.5% and 0.5%, hydrogen peroxide was "moderate" irritative. Since hydrogen peroxide practically loses their microbicidal efficacy in this concentration range under the action of catalase or peroxidase in the presence of blood (Kramer et al. 2008), the active substance is regarded as outdated preventive or therapeutic wound antiseptic (Kramer et al. 2004, 2017). This was confirmed in recent studies which showed that the rate of surgical site infections of contaminated traumatic wounds was significantly more after prophylactic use of hydrogen peroxide 3% when it is compared to Ringer's solution, PVP-iodine and polihexanide 0.04% (Roth et al. 2007, 2017).

Severe CAM reaction was observed after short-term application of Octenilin® (IS=10.2) and chlorhexidine digluconate 0.5% (IS= 9.4) (Table 24). This supports results of Marquart et al. (2010). The special feature of both drugs is strong protein binding capability (Müller and Kramer 2007), which results in a long time of effect for both drugs (Müller and Kramer 2007, Müller et al. 2014). On peritoneal explants, both drugs were more cytotoxic than polihexanide (Kramer et al. 1998). In contrast to the severe irritation in the HET-CAM and the high cytotoxicity for peritoneal explants, Octenilin does not lead to any wound healing inhibition and wounds seem to tolerate it in the same way as Ringer's solution (Eisenbeiss et al. 2012). The biocompatibility index is also as for polihexanide >1 (Müller and Kramer 2008). Octenidine binds immediately to the superficial cells when applied to the wound, and does not reach into the depth due to its low percutaneous absorption (Stahl et al. 2011). On the other hand, if the active substance is placed under pressure in the skin or in the puncture channels as well as in abscess cavities without possible outflow, edematous swelling and tissue damage occurs as a result of active substance remaining on the tissue (Höning et al. 2010). Therefore, the following recommendation for the use of Octenisept® has been

formulated: "To avoid tissue damage, the preparation should not be inserted into the depth of the tissue using syringe. The preparation should only be used for superficial application as swab or spray" (BfArM/PEI 2016). On the other hand, no local necrosis was observed with ensured drainage (Siemers et al. 2011). As a conclusion of the result, agents which induce severe CAM irritation and have a high protein binding are not recommended to be placed under pressure in a skin tissue or inserted into enclosed cavities.

Considering all additional reasons for the rejection of creams on the base of silver sulfadiazine, chlorhexidine, PVP iodine and nitrofural for use as wound antiseptic (Kramer et al. 2017), the irritation displayed onto the CAM supports this assessment displayed by Marquardt et al. (2010) in HET-CAM for PVP-Iodine cream. In contrast to our results the silver sulfadiazine cream induced only slight irritation in the study of Marquardt et al. (2010). Bepanthen® cream was better tolerable than Braunovidon® ointment after 5 minutes, while Furacin® ointment significantly exceeded the irritation of both of them.

For PVP-iodine, the biocompatibility index was determined to be 0.9 when tested with E. coli or 1.0 when tested with S. aureus (Müller and Kramer 2008). In principle, there is nothing to oppose its use. As a result of a systematic review (Vermeulen et al. 2010), PVP-iodine is excluded from the treatment of chronic wounds due to better alternatives. In combination with alcohol, however, PVP-iodine still the antiseptic of choice for puncture, incision and shot injuries due to its penetration into the wound (Kramer et al. 2010, 2017).

In combination with dexpanthenol, chlorhexidine digluconate meets the requirements for an efficient antiseptic (Kramer et al. 2016). As a contraindication, known allergy or anaphylaxis due to chlorhexidine must be considered (Garvey et al. 2007, Moka et al. 2015). It is less effective against Gram-negative bacteria than against Gram-positive bacteria (Kramer et al. 2013). A further disadvantage is the possibility of a plasmid encoded resistance development (Poole 2007, Fraud et al. 2008, Costa 2013).

Silver sulfadiazine (SSD), the active ingredient of Flammazine®, is almost completely ineffective against *S. aureus* and *E. coli* in the presence of 10% fetal calf serum within 30 minutes (Müller and Kramer 2008). Therefore, it is assumed that the efficacy is expected only if the strain is weak. Another disadvantage is the absorption. When applied to burn wounds, silver concentrations were determined to 440 µg/l in blood and to 12 µg/l in urine. As a consequence, monitoring of silver levels in blood and / or urine is recommended when using this active ingredient (Maitre et al. 2002). SSD is

contraindicated in patients who are hypersensitive to SSD. Because sulfonamide therapy is known to increase the possibility of nucleus jaundice, SSD cream should not be used on pregnant women at term, premature newborn and infants during the first 2 months of life. It is also possible that any adverse reaction associated with sulfonamides may occur, such as blood dyscrasias (including agranulocytosis, aplastic anemia, thrombocytopenia, leukopenia and hemolytic anemia) and dermatologic and allergic reactions (including life-threatening cutaneous reactions "Stevens-Johnson syndrome", toxic epidermal necrolysis and exfoliative dermatitis) (Kiker et al. 1977, Jarret et al. 1978, Caffe and Bingham 1982, Blangy et al. 2002). In case of renal insufficiency, the use of SSD is contraindicated, as prolonged topical use of it for pyoderma with gangrenous wounds led to acute renal failure (Chaby et al. 2005). The cytotoxicity of SSD (McCauley et al. 1994, Zapata-Sirvent and Hansbrough, 1993) is believed to be the cause of the retardation of epidermal regeneration in conjunction with more passive signs of a dermatitis-like reaction with spongiosis, parakeratosis, and pseudocarcinoma (Hoekstra et al. 1993). Finally, a strong soluble ointment-protein complex is formed on the wound surfaces, so that no optical wound assessment is possible in the case of burns. As a result, the wound depth cannot be evaluated visually, the exudate remains firmly caked with the wound surface and often leads to a too late indication for required operative care, which subsequently leaves either scars or loss of vital tissue including higher blood loss due to excision. In addition, maceration with the colonization of these wounded areas causes highly resistant wet bacteria such as P. aeruginosa, which are often difficult to control. As a consequence, surgery must be done more frequently in order to prevent serious defects and possibly generalization of the infection. This situation is repeatedly observed in secondary assignments of burn injuries which were treated with SSD for more than 5 days. Therefore, the use of SSD for healing is rather undesirable and it lacks the ability to assess changes in the wound area (Kramer et al. 2004, 2017). The use of SSD for burns is unnecessary because of the low efficacy, the risk of local and systemic side effects including the development of resistance and the presence of better alternatives (Kramer and Richweed 2008a, Kramer et al. 2017).

The active substance nitrofurazone (syn. nitrofural) is only slightly active against *S. aureus* (reduction approx. 1.5 log) and ineffective against *C. albicans*, however effective against *P. aeruginosa* (Hygiene Nord GmbH 2005). Blood and serum significantly reduce the efficacy (Coffey et al. 1991). At the same time, the active substance is highly cytotoxic (Kramer et al. 2008c). According to this, wound healing was significantly delayed by nitrofurazone 0.2% compared to the control in a full-thickness wound model in rats (Saydam et al. 2006). Nitrofurazone is no longer recommended for wound antisepsis because of its insufficient antibacterial effects, the risk of resistance development, the

high sensitization potential, the high cytotoxicity, the lack of quantification for absorption from wounds and the unexplained carcinogenic and teratogenic/ fetotoxic hazard when applied to wounds (Kramer et al. 2004, 2008c).

Wilson and Steck (2000) also used the HET-CAM as a test model in order to examine anti-irritative effects. Due to the absence of observed irritative effects, dexpanthenol and allantoin are both possible additives for antiseptics in order to promote wound healing. Pantothenic acid (vitamin B5) and its biologically active precursor dexpanthenol activate the proliferation of fibroblasts, promote the formation of collagen fibers, stimulate the regeneration of damaged tissue and have an anti-inflammatory and antioxidative effect (Proksch et al. 2002, Oguz et al. 2015, Ulger et al. 2016). Dexpanthenol therefore has been combined in topical antimicrobial preparations, both antiseptics and antibiotics, intended to be used on wounds (Kramer et al. 2016). Additionally, the antiseptic efficacy of chlorhexidine is increased by adding 5% dexpanthenol (Kramer et al. 2016). Animal experiments also displayed promotion of wound healing in animal experiments via regulation of inflammatory response as wells stimulus to fibroblastic proliferation and extracellular matrix synthesis (Jorge et al. 2008, Araujo et al. 2010). Since there seems to be no irritative effect, it is also a possible partner for antiseptics. The irritative effect of octenidine was only slightly reduced by addition of 1.34% dexpanthenol and 0.2 allantoin.

Sodium thiocyanate (NaSCN) 0.698% caused a "slight reaction". NaSCN has an antiphlogistic effect (Kramer and Weuffen 1996, Kramer 2002) and stimulates proliferation of fibroblasts (Machill et al. 1987). Further examinations for the suitability of using NaSCN in combination with wound antiseptics have to be done.

Because sodium thiocyanate particularly promotes the proliferation of rapidly dividing tissues as in spermiogenesis (Gromoll et al. 1990), stimulates hair formation (Kramer et al. 1990a, 1990b, 1996, Minnich et al. 1991, Sima et al. 1995) and proliferation of fibroblasts in cell culture (Adrian et al. 1987, Machill et al. 1987), stimulates wound healing in plant and mammals (Koch 1989), acts antiphlogistically in vitro and in vivo (Kramer and Weuffen 1996), stimulates phagocytosis in wound secretions (Jahr et al. 1986), therefore, the finding that the irritation was reduced from "moderate" to "slight" by the addition of NaSCN to hydrogen peroxide is interesting for any future work.

The small standard deviation of hydrogen peroxide in both concentrations of 1.5% and 0.5% indicates that the results are clustered closely around the mean. The standard deviations of both positive controls, of polihexanide 0.04% in Ringer solution, Dermacyn®, sodium thiocyanate 0.698%, hydrogen peroxide 1.5 % + sodium thiocyanate

0.698% and octenidine 0.05% + panthenol 1.34% + allantoin 0.2% ranged between (1.5-2.61). These values signify accepted dispersions statistically.

Figure 44 shows the percentage of eggs that remained alive after 24 hours, for the negative control and 4 formulations, the range was between 22% and 59%. All the eggs which were tested with the remaining formulations died after 24 hours. Control of bacterial contamination on the eggs after 24 hours was done in order to find if a contamination of the CAM was the cause of eggs death, but no correlation was found. Thus, the explanation of this phenomenon is unclear.

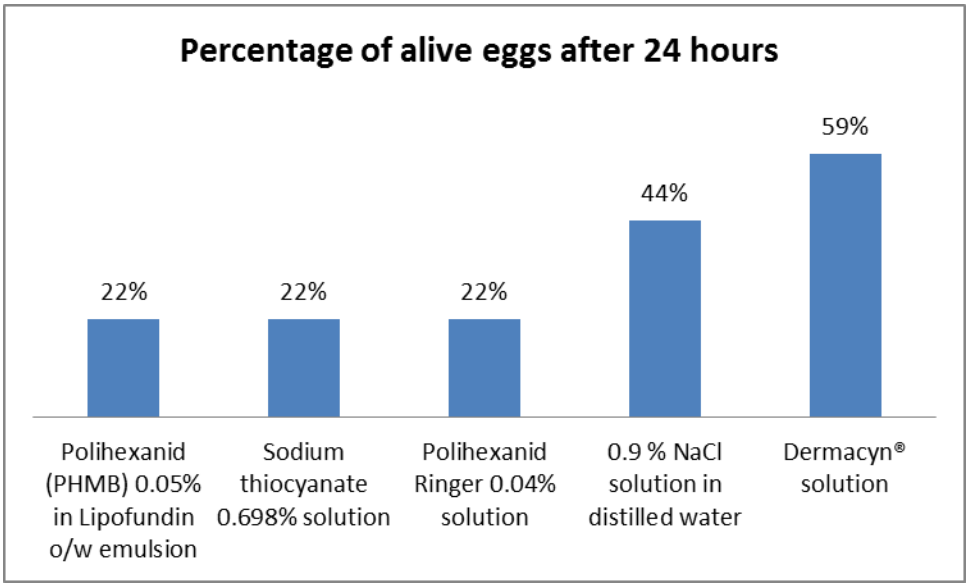

Figure 44: Percentage of living eggs after 24 hours

6 Conclusion

The vascular injuries caused by the tested antiseptics can be considered as an indirect indicator of their wound tolerability. It is suggested that substances with no or low irritation potential on the CAM to be preferred in the clinical practice.

From the tested wound antiseptics, polihexanide and hypochlorite are tolerable without restriction. The same is true for the tested wound oxygenizer.

It appears to be promising to test mixtures of common used antiseptic agents with additives, which could increase the tolerability of the antiseptics, i.e. dexpanthenol, thiocyanate and/ or allantoin, to find more compatible antiseptics with less cytotoxicity.

7 References

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